Designation: F2259 – 10 (Reapproved 2012)ε1

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 F2259; 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 and 4.2 in November 2012.

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 F2064.

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

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E386 Practice for Data Presentation Relating to High-Resolution Nuclear Magnetic Resonance (NMR) Spectroscopy

F2064 Guide for Characterization and Testing of Alginates 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]

3. Terminology

3.1 *Definitions:*

3.1.1 *alginate, n*—polysaccharide obtained from some of the more common species of marine algae, consisting of an insoluble mix of calcium, magnesium, sodium, and potassium salts.

3.1.1.1 *Discussion*—Alginate exists in brown algae as its most abundant polysaccharide, mainly occurring in the cell walls and intercellular spaces of brown seaweed and kelp. Alginate's main function is to contribute to the strength and flexibility of the seaweed plant. Alginate is classified as a hydrocolloid. The most commonly used alginate is sodium alginate. 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

[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.

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 ^1H- and ^{13}C-nuclear magnetic resonance spectroscopy (NMR). A general description of NMR can be found in <761> of the USP 35-NF30. The NMR methodology and assignments are based on data published by Grasdalen et al. (1979, 1981, 1983).[4,5,6] The NMR technique has made it 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.

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.

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

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

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

Nucleus	^{1}H
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 ^{1}H NMR signals. Correct phasing and flat baseline are essential for good results.

6.3 *Calculations:*

6.3.1 ^{1}H 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 ^{1}H 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.

6.3.2 The relations are as follows:

$$G = 0.5(A + C + 0.5(B1+B2+B3))$$
$$M = B4 + 0.5(B1+B2+B3)$$

$$GG = 0.5(A + C - 0.5(B1+B2+B3))$$
$$MG = GM = 0.5(B1+B2+B3)$$
$$MM = B4$$

$$GGM = MGG = (B1)0.5(B1+B2+B3)/(B1+B2)$$
$$MGM = (B2)0.5(B1+B2+B3)/(B1+B2)$$
$$GGG = GG - GGM$$

$$F_G = G/(M+G)$$
$$F_M = M/(M+G)$$

$$F_{\underline{G}G} = GG/(M+G)$$
$$F_{\underline{M}M} = MM/(M+G)$$
$$F_{\underline{G}M} = F_{\underline{M}G} = MG/(M+G)$$

$$F_{G\underline{G}G} = GGG/(M+G)$$
$$F_{M\underline{G}M} = MGM/(M+G)$$
$$F_{G\underline{G}M} = F_{M\underline{G}G} = GGM/(M+G)$$

6.3.2.1 Number average of block length is calculated as previous reported by Grasdalen et al. (1981):[5]

$$N_G = F_G/F_{\underline{G}M}$$
$$N_{G>1} = (F_G - F_{M\underline{G}M})/F_{G\underline{G}M}$$
$$N_M = F_M/F_{\underline{M}G}$$

6.3.2.2 If reducing end signals are integrated ("red-a" and "red-b"), then the estimate of the number average degree of polymerization (DP_n) is:

$$DP_n = (M + G + \text{red-a} + \text{red-b})/(\text{red-a} + \text{red-b})$$

7. Range, Standard Deviation, and Reporting Results

7.1 Data suggest that a suitable value for repeatability and intermediate precision (as measured by the standard deviation,

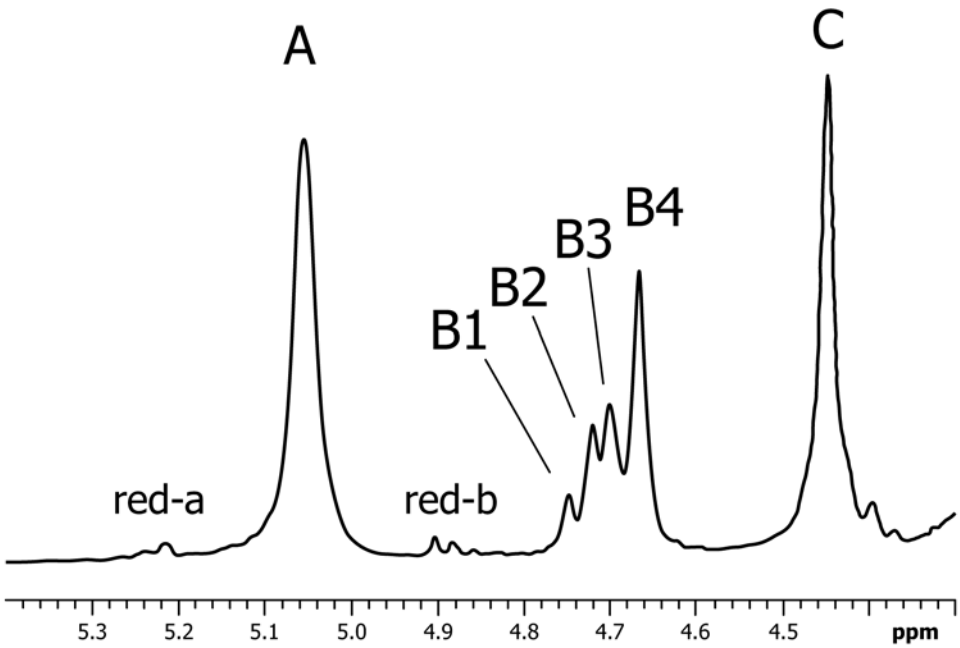

FIG. 1 The Region of the ^{1}H NMR Spectrum of Alginate Used for Quantitative Analysis

SD) for F_G is 0.01. This value applies for all other sequential parameters (monads, diads, and triads) as well. Consequently, sequential parameters should be reported with 2 significant decimals and a standard deviation of 0.01, for example, F_G = 0.68 ± 0.01.

7.2 G-rich alginates should be reported with guluronic acid content as a percentage, for example, "guluronic acid content: 68 %" (standard deviation ±1 %). M-rich alginates should be reported with mannuronic acid content as a percentage, for example, "mannuronic acid content: 66 %" (standard deviation ±1 %).

7.3 For $N_{G>1}$, the overall quality of the data suggests to report a relative standard deviation of approximately 10 %. Consequently, $N_{G>1}$ should be reported with 1 decimal place, and the standard deviation for $N_{G>1}$ should be calculated as 10 % of the measured value, reported with 1 decimal place, for example, $N_{G>1}$ = 13.9 ± 1.4.

7.4 Block lengths N_G and N_M have a relative standard deviation of <5 %.

7.5 When F_G reaches extreme values, an error in quantifying small NMR signals must be considered (F_G > 0.1 or F_G > 0.9). Consequently, the range of the method is considered to span the interval of F_G values from 0.30 to 0.75. If this test method is to be used to characterize alginate anticipated to have an F_G below or above the stipulated interval, then additional validation may be necessary.

7.6 *Non-Applicable Method Parameters:*

7.6.1 *Accuracy*—This parameter is limited by how well the NMR instrument is regularly maintained and controlled. There are no reference samples for a true value of the fraction of guluronate in alginate.

7.6.2 *Specificity*—If there should be any impurities in the sample, unexpected proton signals will be shown in the spectra.

7.6.3 *Linearity*—Not relevant since NMR spectroscopy is quantitative. Each proton NMR peak area is proportional to the number of protons represented by that peak.

7.7 Further recommendations for NMR data presentation can be found in Practice E386.

APPENDIXES

(Nonmandatory Information)

X1. RATIONALE

X1.1 The use of naturally occurring biopolymers for biomedical and pharmaceutical applications and in Tissue Engineered Medical Products (TEMPs) is increasing. This test method is designed to give guidance in the characterization of sodium alginate used in such applications.

X2. BACKGROUND

X2.1 Alginate is a family of non-branched binary copolymers of 1-4 glycosidically linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues (Fig. X2.1). The relative amount of the two uronic acid monomers and their sequential arrangement along the polymer chain vary widely, depending on the origin of the alginate. The uronic acid residues are distributed along the polymer chain in a pattern of blocks, where homopolymeric blocks of G residues (G-blocks), homopolymeric blocks of M residues (M-blocks) and blocks with alternating sequence of M and G units (MG-blocks) co-exist. Thus, the alginate molecule cannot be described by the monomer composition alone. NMR characterization of the sequence of M and G residues in the alginate chain is needed in order to calculate average block lengths. It has also been shown by NMR spectroscopy that alginate has no regular repeating unit.

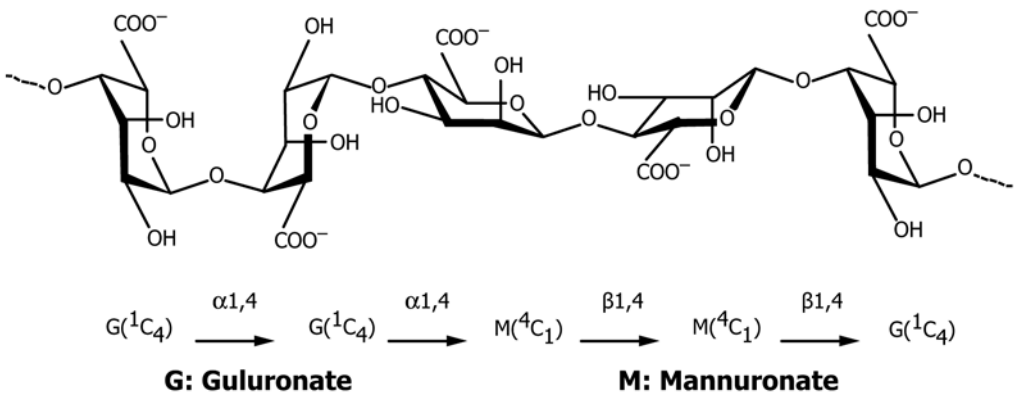

FIG. X2.1 Alginate Structure