Designation: E3181 – 20

Standard Practice for Determination of the Converted Fraction of Starch and Cellulosic Content From a Fuel Ethanol Production Facility[1]

This standard is issued under the fixed designation E3181; 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 practice provides criteria for the sampling, testing, and calculation methodologies used for the quantification of the converted fraction of starch and cellulosic content. Furthermore, this practice covers procedures for the management of the standard error associated with the sampling and testing of before conversion and after conversion samples from a fuel ethanol production facility.

1.1.1 This practice can be used to determine the volume of renewable fuel produced from the simultaneous conversion of starch and cellulosic material eligible for generating D3 RINs under the United States (U.S.) Renewable Fuel Standard (RFS).

1.2 This practice covers the collection and testing of heterogeneous material, including, but not limited to: corn, sorghum, wheat, mash, beer, whole stillage, dried distillers grains with solubles (DDGS), and dried distillers grains.

1.3 This practice is intended to be used in renewable fuel production facilities designed to produce renewable alcohols. Use of this practice in any other type of process has not been reviewed.

1.4 This practice can be utilized using either manual or automatic sampling techniques, so long as the criteria of this practice are followed.

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

1.6 *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]

E122 Practice for Calculating Sample Size to Estimate, With Specified Precision, the Average for a Characteristic of a Lot or Process

E1755 Test Method for Ash in Biomass

E1757 Practice for Preparation of Biomass for Compositional Analysis

E2586 Practice for Calculating and Using Basic Statistics

2.2 *AOAC Standard:*[3]

AOAC 942.05 Ash of Animal Feed

2.3 *Other Publications:*

CFR 40 Code of Federal Regulations (CFR) Part 80 Subpart M Renewable Fuel Standard[4]

EPA-420-B-19-022 Guidance on Qualifying an Analytical Method for Determining the Cellulosic Converted Fraction of Corn Kernel Fiber Co-Processed with Starch[5]

EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR (July, 2014) Additional Detail on the Calculation of the Cellulosic Converted Fraction, and Attribution of Batch RINs for D-code Dependent Feedstocks[5]

3. Terminology

3.1 *Definitions:*

3.1.1 *after conversion sample, n*—a sample obtained from the process after the conversion process of starch and cellulosic content is determined to be complete as defined by the producer's reaction standards.

3.1.2 *amyloglucosidase, n*—an enzyme that specifically catalyzes the hydrolysis of α-D-glucosidic bonds successively from the non-reducing ends of oligo-and polysaccharides with the release of β-D-glucose.

[1] This practice is under the jurisdiction of ASTM Committee E48 on Bioenergy and Industrial Chemicals from Biomass and is the direct responsibility of Subcommittee E48.05 on Biomass Conversion.

Current edition approved Feb. 1, 2020. Published March 2020. DOI: 10.1520/E3181-20.

[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 AOAC International, 2275 Research Blvd., Suite 300, Rockville, MD 20850-3250, http://www.aoac.org.

[4] Available from the U.S. Government Printing Office, Superintendent of Documents, 732 N. Capitol St. N.W. Mail Stop: SDE Washington, DC 20401.

[5] Available from the United States Environmental Protection Agency (EPA), 1200 Pennsylvania Avenue, N.W., Washington, DE 20460, www.epa.gov.

3.1.3 *ash, n*—inorganic residue remaining after combustion, determined by definite prescribed methods.

3.1.3.1 *Discussion*—Test Method E1755 and AOAC 942.05 are acceptable prescribed methods.

3.1.4 *backset, n*—a recycled liquid stream that also contains some level of dissolved and suspended solids used as makeup water in a renewable fuel production facility.

3.1.5 *beer, n*—the mash after it has undergone fermentation and has been deemed complete by virtue of the carbohydrates being converted into renewable fuel by enzymes and fermenting organisms.

3.1.6 *before conversion sample, n*—a sample obtained from the process before any starch or cellulosic content conversion has taken place.

3.1.7 *cellulose, n*—a crystalline, straight chain glucan with β(1→4) linkages.

3.1.8 *cellulosic content, n*—as defined by EPA RFS documentation: the sum of cellulose, hemicellulose, and lignin in cellulosic feedstock.

3.1.9 *cellulosic feedstock, n*—any feedstock composed predominately of cellulose, hemicellulose, or lignin.

3.1.9.1 *Discussion*—The EPA allows renewable fuel produced from cellulosic feedstocks with an average adjusted cellulosic content of 75 % (on a dry mass basis) to be eligible for the generation of the D3 RIN code.

3.1.9.2 *Discussion*—For example, in a facility producing ethanol from corn, the EPA considers the corn kernel fiber to be a cellulosic feedstock.

3.1.10 *certification run, n*—a group of converted fraction determinations to establish the converted fraction of starch and cellulosic content in a process that involves the simultaneous conversion of feedstocks that are predominantly cellulosic and feedstocks that are not predominantly cellulosic.

3.1.11 *converted fraction (CF), n*—a unitless average mass fraction representing the portion of the feedstock converted to either cellulosic or non-cellulosic fuel.

3.1.12 *DDG, n*—the dried whole stillage; typically sold as an animal feed component.

3.1.13 *DDGS, n*—the dried whole stillage with solubles; typically sold as an animal feed component.

3.1.14 *feedstock, n*—any raw material used as a carbohydrate source for fermentation.

3.1.15 *GHG, n*—greenhouse gas; greenhouse gases include: carbon dioxide, methane, nitrous oxide, and fluorinated gases.

3.1.16 *glucan, v*—refers to any polymer of glucose, regardless of structure.

3.1.17 *hemicellulose, n*—a polymeric, branched carbohydrate with mixed C5 and C6 monomeric sugars, typically with a xylose or mannose backbone.

3.1.17.1 *Discussion*—For the purpose of this practice, C5 sugars are five carbon sugars derived from hemicellulose. C6 sugars are six carbon sugars derived from either starch, hemicellulose, or cellulose.

3.1.18 *lignin, n*—a complex organic polymer with aromatic components; non-carbohydrate found in cellulosic feedstock.

3.1.19 *makeup water, n*—any liquid stream added to the process to prepare the mash slurry in a renewable fuel production facility.

3.1.20 *mash, n*—a mixture or slurry of water (including recycled water streams) and ground grain.

3.1.21 *monosaccharide, n*—any of the class of sugars (for example, glucose) that cannot be hydrolyzed to give a simpler sugar.

3.1.22 *oligosaccharide, n*—a carbohydrate whose molecules are composed of a relatively small number of monosaccharide units.

3.1.23 *polysaccharide, n*—a carbohydrate (for example, starch, cellulose, or glycogen) whose molecules consist of a long chain of sugar molecules bonded together.

3.1.24 *predominantly cellulosic, adj*—feedstock that has an average adjusted cellulosic content of 75 %, measured on a dry mass basis; furthermore, this ‘‘adjusted cellulosic content’’ is the percent of organic (non-ash) material that is cellulose, hemicellulose, or lignin (CFR 40, Part 80 and EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR).

3.1.25 *Renewable Fuel Standard (RFS), n*—program authorized under the Energy Policy Act of 2005 and expanded under the Energy Independence and Security Act of 2007; it requires an increasing amount of renewable fuel to be blended into the U.S. liquid fuel supply each year through the year 2022.

3.1.26 *resistant starch, n*—starch that cannot be readily monomerized by commercially available alpha amylase and glucoamylase; this type of starch can be caused by several different factors: physical inaccessibility, molecular structure configuration, cross linkage, and lipid binding.

3.1.26.1 *Discussion*—When resistant starch is suspected to be present, it is important that the starch measurement technique used be designed to access resistant starch. Many starch procedures have options that include, for example, dimethyl sulfoxide (DMSO) or potassium hydroxide (KOH) to ensure complete access and hydrolysis of any resistant starch present.

3.1.27 *RINs, n*—Renewable identification numbers (RINs) are unique numbers generated to represent a volume of renewable fuel used for compliance to the RFS program.

3.1.27.1 *D3 RINs, n*— the RIN code assigned to gallons of renewable fuel produced from any cellulose, hemicellulose, or lignin that has lifecycle greenhouse gas emissions that are at least 60 % less than the baseline lifecycle greenhouse gas emissions.

3.1.27.2 *D6 RINs, n*—the RIN code assigned to gallons of renewable fuel produced from renewable biomass and has lifecycle greenhouse gas emissions that are at least 20 % less than baseline lifecycle greenhouse gas emissions, unless the fuel is exempt from this requirement.

3.1.28 *starch, n*—a polysaccharide consisting of glucose monomers joined in α 1,4 linkages; the simplest form of starch is the linear polymer amylose, while amylopectin is the branched form.

3.1.28.1 *Discussion*—For the purposes of this practice, starch shall include the soluble C6 glucan mono- and oligosaccharides. Since the EPA definition for "cellulosic content" is specific to the "sum of cellulose, hemicellulose, and lignin in cellulosic feedstock," the soluble C6 glucan mono- and oligosaccharides shall be included with the starch total (CFR 40, Part 80). Since the only difference between starch and cellulose is the orientation of the 1, 4 linkage, to determine the starch content of a sample that contains both starch and cellulose, an assay that utilizes amyloglucosidase is necessary.

3.1.29 *total solids, n*—the amount of total suspended and dissolved matter contained in a sample.

3.1.30 *whole stillage, n*—the beer once the renewable fuel has been removed.

4. Significance and Use

4.1 In 2014, the U.S. EPA published the final rules adding renewable fuel pathways under the RFS Program. The rules qualified kernel fiber as a cellulosic feedstock meeting the 60 % greenhouse gas (GHG) reduction and qualifies for the generation of D3 RINs. These rules allow for two approaches for kernel fiber conversion (CFR 40, Part 80 and EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR):

4.1.1 Producers of cellulosic fuels derived from conversion of feedstocks that are predominantly cellulosic, where "predominantly cellulosic" is defined as feedstock that has an average adjusted cellulosic content of 75 %, measured on a dry mass basis; furthermore, this "adjusted cellulosic content" is the percent of organic (non-ash) material that is cellulose, hemicellulose, or lignin (CFR 40, Part 80 and EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR).

4.1.2 Producers of cellulosic fuels derived from the simultaneous conversion of feedstocks that are predominantly cellulosic and feedstocks that are not predominantly cellulosic (CFR 40, Part 80 and EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR).

4.2 Producers that wish to gain approval to the pathway that claims simultaneous conversion of feedstocks that are predominantly cellulosic and feedstocks that are not predominantly cellulosic are required to quantify the amount of renewable fuel that is derived specifically from cellulosic content and from starch. To accomplish this, the producer needs to quantify the amount of cellulosic content and starch present before the conversion process begins and after the conversion process is complete. These measurements of cellulosic content and starch content before and after conversion are used to calculate a converted fraction of each, which is then used to ratio the renewable fuel produced accordingly and assign those respective gallons the D6 or D3 RIN code (CFR 40, Part 80 and EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR).

5. Test Method Requirements

5.1 Any test method used for the quantification of starch and cellulosic content and the related converted fraction of each shall be evaluated for both accuracy and precision.

5.1.1 Agreement of the test method for cellulosic content may be demonstrated by showing agreement of the test method for cellulosic content to a commercially available reference material[6] with established and documented concentrations of cellulosic content. When the commercially available reference material is analyzed, the test method shall achieve a mean test value that falls within 20 % of the reported cellulosic content value (EPA-420-B-19-022) as documented with the commercially available reference material. Test values that fall within 20 % of the reported cellulosic content value are deemed reasonably accurate.

5.1.1.1 *Discussion*—Producers generating RINs with a D code of 3 or a D code of 7 using two or more different feedstocks (at least one of which does not have at least 75 % average adjusted cellulosic content) which are processed simultaneously through an in situ biochemical hydrolysis treatment will similarly have additional registration requirements to help ensure that cellulosic RINs are being generated accurately. At the time of registration, such a producer must submit: *(1)* the overall fuel yield, including supporting data demonstrating this yield and a discussion of the possible variability in overall fuel yield that could be expected between reporting periods; *(2)* the cellulosic converted fraction that will be used for generating RINs under § 80.1426(f)(3)(vi); including chemical analysis data (described in more detail below) supporting the calculated cellulosic converted fraction and a discussion of the possible variability that could be expected between reporting periods; and *(3)* a description of how the cellulosic converted fraction is determined and calculations showing how the data were used to determine the cellulosic converted fraction. Data used to calculate the cellulosic converted fraction by producers using in situ biochemical hydrolysis treatment who seek to generate cellulosic RINs must be representative and obtained using an analytical method certified by a voluntary consensus standards body (VCSB) or using a non-VCSB method that would produce reasonably accurate results. If using a non-VCSB approved method to generate the data required to calculate the cellulosic converted fraction for a given fuel, then the producer will need to show that the method used is an adequate means of providing reasonably accurate results by providing peer reviewed references to the third-party engineer performing the engineering review at registration. A full description of the formulas in § 80.1426(f)(3) used to calculate RINs for renewable fuel described by two or more pathways, including methods used to calculate the converted fraction, can be found in the associated memo to the docket (CFR 40, Part 80).

5.1.2 It has been demonstrated that using sample preparation drying temperatures above 65 °C when drying and preparing samples for analysis can cause the formation of resistant starch which can lead to the generation of erroneous results. For this reason, samples that need to be dried prior to analysis shall follow the guidance of Practice E1757.

5.1.3 It is critical that any starch method used for the determination of the converted fraction of starch for the purposes of this practice be designed to access resistant starch. Many starch procedures have options that include, for example,

[6] A possible source of reference materials is NIST (National Institute of Standards and Technology).

dimethyl sulfoxide (DMSO) or potassium hydroxide (KOH) to ensure complete access and hydrolysis of all starch present.

5.1.4 Precision of the test methods shall be established and documented by quantifying the reproducibility of the test methods for starch and cellulosic content. To determine the reproducibility of the test methods, six replicates of one prepared sample of representative substrate shall be analyzed. The standard deviation of the test method shall be calculated and documented (this is the uncertainty of the testing method).

6. Sample Collection Requirements

6.1 The number of subsamples needed to achieve a representative composite sample shall be determined by using Practice E122.

6.2 The purpose of this section and the use of Practice E122 is to ensure that the resulting composite sample is representative of the material in the reaction vessel. For this purpose, a total solids test can be used as the test data for the purposes of Practice E122. Total solids can be a surrogate for starch and cellulosic content as it relates to homogeneity testing since both starch and cellulosic content represent insoluble materials.

6.3 Statistically, increasing sampling size will make the sample mean closer to the population mean. According to Central Limit Theorem, if you are sampling from a normal population, a small sample size (for example, $n = 6$) may be sufficient; however, if the population distribution is highly skewed, you will need a much larger sample size (for example, $n = 25$ or higher).[7]

6.4 When using Practice E122, the user must choose the number of the standard deviations multiplier and the maximum acceptable relative difference between the true average and the sample average, E. The example data set uses 3 for the standard deviation multiplier and 2.0 for the maximum acceptable relative difference between the true average and the sample average. Using 3 and 2.0, respectively, is recommended for this practice; however, more stringent parameters may be merited to manage the variation in the reported results.

6.5 An example data set using Practice E122 is included in Appendix X1.

6.6 Sample volume of the subsamples and total composite sample will be determined and is dependent upon the needs of the analytical measurement.

7. Sample Analysis

7.1 The number of analysis replications performed on each sample may be managed to sufficiently lower the standard error of the results to meet the required variation criteria for the converted fraction determination.

7.1.1 The formula for the standard error (SE) of a test result is as follows:

$$SE = \frac{\sigma}{\sqrt{n}} \quad (1)$$

where:

σ = the standard deviation of the sample results, and
n = the number of sample results.

7.1.1.1 The standard error goes down by a factor of the square root of the increase in the number of sample analysis replicates. The variation in the results, therefore, goes down by 50 % for every fourfold increase in the number of sample analysis replicates performed. See Appendix X2 for an example of the reduction in standard error experienced in proportion to the number of sample replications performed.

7.1.2 The number of replications on each sample may be increased to the extent necessary to lower the standard error of the result reported.

7.1.3 An example data set demonstrating the reduction in standard error using multiple analytical replications is included in Appendix X2.

8. Converted Fraction Calculation

8.1 The U.S. EPA has specific requirements that apply to producers that seek to generate cellulosic D3 RINs for fuel produced by biochemical hydrolysis treatment where cellulosic and non-cellulosic components of feedstocks are simultaneously hydrolyzed to fermentable sugars (for example, corn starch and a crop residue). These requirements include the determination of the converted fraction of starch and cellulose content. The converted fractions of starch and cellulosic content are then used to calculate the percentage of renewable fuel gallons that were derived from starch and the percentage of gallons that were derived from cellulosic content (CFR 40, Part 80 and EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR). A further explanation and examples of how the converted fractions may be calculated are included in Appendix X5 and Appendix X6.

8.1.1 The U.S. EPA regulations require that all determinations of the converted fraction of starch and cellulosic content used to determine the gallons of renewable fuel eligible for the generation of D3 RINs be reviewed and approved by a third-party engineering firm (CFR 40, Part 80 and EPA-HQ-OAR-2012-0401; FRL-9910-40-OAR).

9. Converted Fraction Statistical Confidence Criteria

9.1 The P-value shall be determined for the converted fraction of cellulosic content obtained using all the cellulosic content converted fraction results for any given certification run. This P-value will determine if the variation is sufficiently low to state with statistical confidence that the converted fraction of cellulosic content is greater than zero.

9.1.1 The P-value is set by the user to match the data quality objective. Typical values are 0.1, 0.05, or 0.025. The P-value threshold for statistical confidence for this practice is set at a maximum of 0.05. The test statistic (T) shall be calculated as follows:

$$T = \frac{\mu}{\sigma / \sqrt{n}} \quad (2)$$

where:

μ = the mean of the sample results from the certification run,

[7] Johnson, R. A, Miller, I., and Freund, J., *Miller and Freund's Probability and Statistics for Engineers*, 6th edition, p. 208–209.

σ = the standard deviation of the sample results from the certification run, and
n = the number of sample results in the certification run.

9.2 The P-value shall be calculated for an n-1 distribution. Reference Practice E2586 for calculating P-value.

9.3 An example data set showing a passing P-value is included in Appendix X3.

10. Variation Criteria for the Reported Percentage of D3 RINs

10.1 The converted fractions of starch and cellulosic content shall be used to calculate the percentage of renewable fuel produced that can be assigned a D3 and D6 RIN code. These calculations are prescribed in 40 CFR §80.1426.

10.1.1 The Coefficient of Variation (CV) of the reported percentages of D3 gallons produced for each certification run shall be determined in accordance with the following equation:

$$CV = \frac{\sigma}{\mu} \quad (3)$$

where:

σ = the standard deviation of the sample results from the certification run, and
μ = the mean of the sample results from the certification run.

10.2 Example data sets showing certification runs assessed using the Coefficient of Variation are included in Appendix X4.

11. Keywords

11.1 beer; cellulosic; cellulosic content; converted fraction; corn; D3 RINs; Dried Distillers Grains with Solubles; Environmental Protection Agency; ethanol; fiber; heterogeneous substrates; mash; renewable fuel; Renewable Fuels Standard; sorghum; starch; wheat; whole stillage

APPENDIXES

(Nonmandatory Information)

X1. EXAMPLE DATA SET USING PRACTICE E122

X1.1 Table X1.1 shows an example data set when using Practice E122 as described in Section 6. The example data set shows total solid results for 24 samples taken from a fermentation vessel. The example data set uses 3 for the standard deviation multiplier and 2.0 for the maximum acceptable relative difference between the true average and the sample

TABLE X1.1 Example Data Set for Practice E122

Subsample #	Analytical Result
1	27.16
2	27.09
3	27.41
4	27.85
5	28.37
6	28.82
7	26.40
8	27.67
9	27.49
10	27.94
11	29.12
12	29.42
13	28.53
14	27.62
15	27.60
16	26.34
17	27.88
18	27.62
19	28.04
20	29.30
21	27.58
22	29.52
23	27.42
24	27.17
Test Set Mean, μ	27.89
Test Set Standard Deviation, σ	0.87
Number of Standard Deviations Multiplier	3
The maximum acceptable relative difference between the true average and the sample average, *E*	2.0 %
Number of Subsamples, *n*, needed to meet desired Precision, *E*, at the confidence interval determined by the standard deviations multiplier	21.8

average as recommended in this practice. In this example, the number of subsamples required to be taken and included in the composite sample to meet the desired accuracy criteria is 22.

X2. EXAMPLE DATA SET DEMONSTRATING THE REDUCTION IN STANDARD ERROR USING MULTIPLE ANALYTICAL REPLICATIONS

X2.1 Table X2.1 and Fig. X2.1 show the expected reduction in standard error based on the number of sample replications performed as described in Section 7. The number of sample replications can be increased until the desired reduction in error is achieved.

TABLE X2.1 Example of Standard Error Reduction

# of Sample Analysis Replicates per Sample	Variation in the Results	% Reduction in Standard Error
1	1	0.0
4	0.5	50.0
16	0.25	75.0
64	0.125	87.5
256	0.0625	93.8
1024	0.03125	96.9
4096	0.015625	98.4