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Certified Reference Materials

AOCS 0919-A

Report of the certification process for

Non-modified

Maize Certified Reference Materials

First Batch

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Abstract

This report describes the preparation and certification of the maize Certified Reference Material (CRM) AOCS 0919-A produced by AOCS Technical Services in 2019. The CRMs have been prepared at AOCS according to ISO 17034:2016 and are intended to serve as control material for third party testing of maize for transformation events. Non-modified maize seed powder (Breeding line: NP2222/NP2391) was provided by Syngenta Crop Protection, LLC and was prepared by grinding the bulk source at AVEKA Inc, Woodbury, MN (an ISO 9001:2015 accredited facility). The non-modified maize seed powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association (an ISO 17025:2017 accredited facility). The certified value of non-modified CRM AOCS 0919-A was based on the purity of the bulk seed and is 0 g/kg. Homogeneity testing was performed at Eurofins-GeneScan, New Orleans, LA (an ISO 17025:2005 accredited laboratory) using quantitative real-time PCR after the CRM AOCS 0919-A was bottled. Homogeneity results indicated that CRM AOCS 0919-A is homogeneous and were used to verify the absence of Events MIR162, MZHG0JG and MZIR098 in this CRM. CRM AOCS 0919-A is available in 27-ml glass headspace vials. This CRM shall be stored dry in a sealed container at ambient or cooler conditions in the dark.

Acknowledgements

The authors would like to express sincere appreciation and gratitude to several individuals and their companies for support and guidance throughout this project. Thanks go to Kristina Burgin, Syngenta Crop Protection, LLC, for offering AOCS the opportunity to manufacture and distribute these products; to Sandra Harrison and Charlie Drennan at Illinois Crop Improvement Association for packaging the samples; and to Frank Spiegelhalter, Greg Ditta, E. Pearce Smith, and Daniel Thompson, Eurofins-GeneScan for event-specific, PCR analysis including the provision of information on running the analyses and interpreting the results.

Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	A related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food
Cycle threshold (Ct)	Number of PCR cycles required for the fluorescent signal to cross a threshold that exceeds background level
DNA	Deoxyribonucleic Acid is the linear, double-helix macromolecule that makes up the genetic material of most organisms
Detection Limit	Lowest level at which target DNA can be detected in a sample.
EC	European Commission
Genome	The full set of genes and associated DNA characteristic of an organism
GMO	Genetically modified/engineered organism: an organism in which the genetic material has been changed through modern biotechnology in a way that does not occur naturally by multiplication and/or natural recombination.
ISO	International Organisation for Standardisation
PCR	Polymerase Chain Reaction: technique used to determine whether a sample of plant tissue contains a particular DNA sequence. PCR relies on primer sets that bind to a particular target DNA sequence and a special DNA-copying enzyme (DNA polymerase) that exponentially amplifies the target sequence for identification and measurement

Qualitative PCR	PCR methods that determine the presence or absence of a specific target DNA sequence at a particular level of detection
Quantitation Limit	Lowest level at which the amount of target DNA sequence in a sample can be reliably quantitated
Quantitative PCR	PCR methods that estimate the relative amount of target DNA sequence in a mixture of DNA molecules
RSDr	Relative standard deviation
SD	Standard deviation

Introduction

Plant biotechnology is an extension of traditional plant breeding. It allows plant breeders to develop crops with specific traits including insect, disease, and herbicide resistance; processing advantages; and nutritional enhancement. An important component for identifying these new traits is a Certified Reference Material (CRM) created from leaf, seed, or grain containing the new trait as well as a CRM created from the conventionally bred matrix. The European Commission has mandated that from 18 April 2004, a method for detecting a new event derived from transgenic technology and Certified Reference Material must be available before the EC will consider authorization of a new crop derived from transgenic technology. Several nations outside Europe also require grain and ingredients to be labeled above a threshold level before accepting a shipment.

To meet the above regulatory requirements for GMO determination, CRM AOCS 0919-A was manufactured from maize seed according to ISO 17034:2016 and in accordance with EC No 1829/2003, EC No 641/2004 and EC No 619/2011. This CRM is available from AOCS.

Material Preparation and Particle Size Analyses

The non-modified maize breeding line NP2222/NP2391 was used in the production of AOCS CRM 0919-A. Seed from NP2222/NP2391 maize was first milled and analyzed for particle size distribution at AVEKA, Inc., Woodbury, Minnesota (an ISO 9001:2015 accredited facility).

Bulk seed received by AVEKA, Inc. from Syngenta Crop Protection, LLC was milled in a Fitzmill cryogenic hammermill using first a 690 µm screen. To further reduce particle size, this ground material was milled again under the same conditions using a 510 µm screen. The material was blended in a Patterson-Kelley V-blender, and after homogenization six samples taken at random were subject to particle size analyses using a Horiba LA-950 Light Scattering Particle Analyzer. For each sample, the particle size mean and range, and the percentage of particles below a certain size was calculated (Table 1). On average, the particle size of CRM AOCS 0919-A was 130.20 $\pm$ 4.31 µm, and

90% of the particles were smaller than 266.32 ± 12.25 µm.

Table 1. Results of Particle Size Analyses of CRM AOCS 0919-A Conducted by AVEKA, Inc.

	Sample 1 (µm)	Sample 2 (µm)	Sample 3 (µm)	Sample 4 (µm)	Sample 6 (µm)	Sample 6 (µm)	Average (µm)	Standard Deviation (µm)
Mean	125.59	126.54	132.52	137.22	130.93	128.40	**130.20**	**4.31**
Range	0.88-394	0.88-394	0.88-517	0.88-452	0.88-517	0.88-394	N/A[(a)]	N/A
D10[(b)]	18.07	18.27	17.12	18.70	17.52	17.32	17.83	0.61
D50[(b)]	119.65	115.27	117.83	125.47	115.23	119.65	118.85	3.80
D90[(b)]	249.62	258.27	275.93	281.29	273.11	259.70	**266.32**	**12.25**

(a) N/A = not applicable

(b) D10, D50 and D90 indicate that 10%, 50% or 90% of the particles, respectively, are smaller than size given in table

The particle size distribution for each of the samples analyzed is presented as a histogram, with the x-axis showing discrete size bins up to 517.2 µm (Figure 1). Figure 1-A represents the percentage of particles of a given size, and Figure 1-B represents the cumulative particle size distribution, which reflects the total percentage of particles smaller than a given size. For all samples analyzed, 100% of particles were ≤ 517.2 µm.

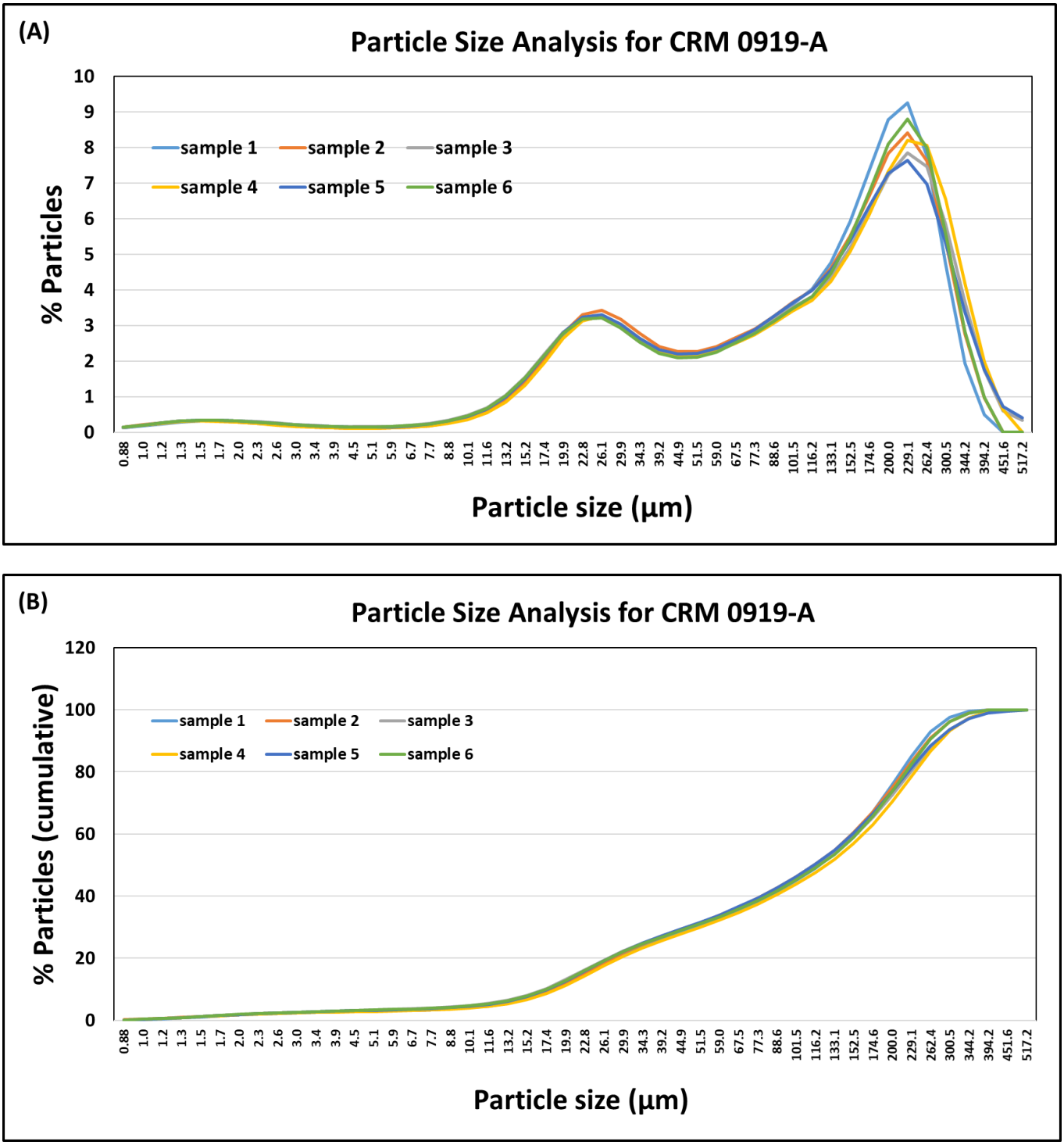

Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes

Bulk, non-modified maize seed powder for the production of CRM AOCS 0919-A was

delivered to AOCS and it was then aliquoted and packaged in 27-ml glass headspace vials and sealed under a nitrogen gas environment at the Illinois Crop Improvement Association (an ISO 17025:2017 accredited facility).

Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce AOCS 0919-A was assessed by Syngenta Crop Protection, LLC. A total of 7200 seeds (24 pools of 300 seeds/pool) of maize breeding line NP2222/NP2391 were subjected to individual seed testing for the presence of MIR162, MZHG0JG and MZIR098 maize by qualitative real-time PCR. None of the NP2222/NP2391 maize seed pools tested positive for MIR162, MZHG0JG or MZIR098 maize.

Purity estimation was calculated using SeedCalc8 (Remund *et al*., 2008). The % impurity in the sample was 0% when 7200 seeds were tested.

The measurement uncertainty is the expanded uncertainty using the value of the upper bound of impurity at 9 g/kg. The standard uncertainty can be obtained by dividing the expanded uncertainty by $2\sqrt{3}$ (rectangular distribution).

The standard uncertainty for AOCS CRM 0919-A is 0.1 g/kg.

Homogeneity Testing

The material used for the production of CRM AOCS 0919-A, non-modified maize, contains 0% impurities and is expected to be homogenous. After NP2222/NP2391 seed was ground and bottled, ten samples of AOCS CRM 0919-A were randomly selected using the Microsoft Excel Random Number Generator function and were sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025:2005 accredited laboratory) for homogeneity testing using quantitative real-time PCR.

Homogeneity of CRM AOCS 0919-A was based on the quantification of the endogenous *adh1* maize gene using an *adh1*-specific, quantitative real-time PCR (http://gmo-crl.jrc.ec.europa.eu/summaries/MIR162_validated_Method.pdf). A total of 10 samples were analyzed, and for each sample, 2 independent DNA extractions and quantifications were

performed at Eurofins-Genescan using a test portion of 1 gram. Extracted DNA was checked for integrity by gel-electrophoresis and quantified prior to using it in quantitative real-time PCR. For each of the DNA extracts, all PCR reactions were done in triplicate. The absence of Events MIR162, MZHG0JG and MZIR098 in CRM AOCS 0919-A were assessed using event-specific, quantitative real-time PCR methods (http://gmo-crl.jrc.ec.europa.eu/summaries/MIR162_validated_Method.pdf; https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-16-VR.pdf; https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-17-VR.pdf.

The cycle threshold (Ct) values for an endogenous *adh1* maize gene and for event MIR162, MZHG0JG or MZIR098 were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between event MIR162, MZHG0JG or MZIR098 copy number and *adh1* copy number (MIR162, MZHG0JG or MZIR098 cp#/*adh1* cp#) was calculated and used to estimate the within-unit relative standard deviation ($\mathrm{RSD_w}$) and between-unit relative standard deviation ($\mathrm{RSD_b}$).

Within-unit relative standard deviation ($\mathrm{RSD_w}$), between-unit relative standard deviation ($\mathrm{RSD_b}$) were calculated as:

Within-unit RSD: $RSD_w = \frac{\sqrt{MS_{within}}}{\bar{y}}$

Between-unit RSD: $RSD_b = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$

where,

$\mathrm{MS_{within}}$ within-unit mean square from an ANOVA
$\mathrm{MS_{between}}$ between-unit mean square from an ANOVA
$\bar{y}$ mean of all results of the homogeneity study
n mean number of replicates per unit

Table 2. The within-unit relative standard deviation (RSD_w), and the between-unit relative standard deviation (RSD_b) for vials of AOCS 0919-A.

CRM	RSD_w [%]	RSD_b [%]
AOCS 0919-A	Below LOQ	Below LOQ

The CRM will be determined to be homogeneous if the within-unit relative standard deviation ($\mathrm{RSD_w}$) and between-unit relative standard deviation ($\mathrm{RSD_b}$) are both ≤20Based on the quantitative real-time PCR analyses conducted, it was concluded that CRM AOCS 0919-A is homogenous (Table 2) because all results are below LOQ. These results are in agreement with homogeneity results from qualitative real-time PCR analyses and with the purity estimate for material non-modified maize calculated in the Certified Value and Measurement Uncertainty section above.

Trait Verification

The absence of Events MIR162, MZHG0JG and MZIR098 in non-modified NP2222/NP2391 maize material was assessed in the same ten AOCS CRM 0919-A samples that were analyzed for homogeneity using event-specific quantitative PCR analysis. Quantitative results were converted to qualitative data, and the results are presented in Table 3. In all instances Events MIR162, MZHG0JG and MZIR098 were absent.

Table 3. Qualitative results for the verification of CRM AOCS 0919-A non-modified maize samples as tested by Eurofins-GeneScan with event-specific, quantitative PCR analysis.	
Sample	**Events MIR162, MZHG0JG and MZIR098 Presence**
AOCS 0919-A 73	Negative
AOCS 0919-A 94	Negative
AOCS 0919-A 173	Negative
AOCS 0919-A 220	Negative
AOCS 0919-A 229	Negative
AOCS 0919-A 248	Negative
AOCS 0919-A 250	Negative
AOCS 0919-A 311	Negative
AOCS 0919-A 344	Negative
AOCS 0919-A 449	Negative

Stability

Time, temperature and light are regarded as the most relevant influences on the stability of CRM (Linsinger, et al., 2001). The influence of light is mitigated by shipping and storing the vials in boxes, thus minimizing the possibility of degradation due to light. The influence of temperature is mitigated by storing the vials in a temperature-controlled room, and shipping vials at ambient temperature.

Stability of these CRMs has been listed as 1 year from the certification date. The materials were processed and are stored at ambient temperature, under nitrogen gas, in 27 -mL glass headspace vials. These materials are expected to be stable for longer than the estimated expiration date. The stability of the powder material will be reevaluated at time of expiration. If the samples are determined to be stable, the certificates will be extended.

References

AVEKA, Inc.; 2045 Wooddale Drive. Woodbury, MN 55125; Telephone : +1 651 730 1729; https://www.aveka.com/

Biosafety Clearing House Living Modified Organism (LMO) Registry http://bch.cbd.int/database/lmo-registry/

Eurofins-GeneScan; 2219 Lakeshore Drive, Suite 400, New Orleans, LA 70122; Telephone: +1 504 297 4330 Toll Free: +1 866 535 2730 Fax: +1 504 297 4335 https://www.eurofinsus.com/food-testing/testing-services/gmo/

Illinois Crop Improvement Association, 3105 Research Road, Champaign, IL 61826; Telephone: +1 217 359 4053 Fax: +1 217 359 4075; https://www.ilcrop.com/

ISO 9001:2015, Quality Management Systems – Requirements

ISO 17025:2005 and ISO 17025:2017, General Requirements for the Competence of Testing and Calibration Laboratories

ISO 17034:2016, General Requirements for the Competence of Reference Material Producers

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Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32003R1829&from=en)

Remund K, Simpson R, Laffont J-L, Wright D, and Gregoire S. Seedcalc8. 2008. (https://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--3449--1102.html)