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Certified Reference Materials

AOCS 0224-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 0224-A produced by AOCS Technical Services in 2024. The CRMs have been prepared by 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 was provided by Syngenta Seeds, LLC and was prepared by grinding the bulk seed 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 0224-A was based on the purity of the bulk seed material and is 0 g/kg. Homogeneity testing was performed at Eurofins-GeneScan GmbH, Freiburg, Germany (an ISO 17025:2005 accredited laboratory) using quantitative and qualitative real-time PCR after the CRM AOCS 0224-A was bottled. Homogeneity results indicated that CRM AOCS 0224-A is homogenous. MZIR260 DNA was below the practical limit of quantification (pLOQ). CRM AOCS 0224-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 personnel from Seeds Product Safety Syngenta for offering AOCS the opportunity to manufacture and distribute these products; to AVEKA, Inc. for processing the samples, to Illinois Crop Improvement Association for packaging the samples; and to Eurofins-GeneScan for event-specific, real-time PCR analysis including the provision of information on running the analyses and interpreting the results.

Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	Conventional counterpart means 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 (Cq)	Number of PCR cycles required for the fluorescent signal to cross a threshold that exceeds background level
Detection Limit	Lowest level at which target DNA can be detected in a sample.
DNA	Deoxyribonucleic Acid is the linear, double-helix macromolecule that makes up the genetic material of most organisms
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
LOD	Limit of Detection
LOQ	Limit of Quantification
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
RSD_r	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 analytical requirements for GMO determination, CRM AOCS 0224-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 Processing and Particle Size Analyses

The non-modified maize hybrid IJ7010/AX5707 was used in the production of CRM AOCS 0224-A. Syngenta Seeds, LLC delivered approximately 5 kg of non-modified maize seed to AVEKA Inc., Woodbury, Minnesota (an ISO 9001:2015 accredited facility).

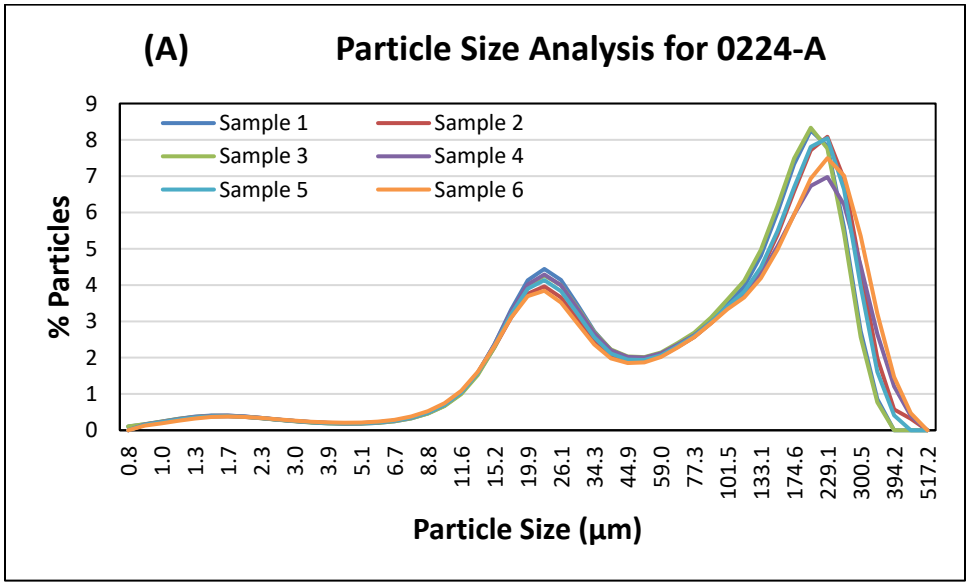
Bulk seed was milled at AVEKA, Inc. in a Fitzmill cryogenic hammermill using a 510 µm screen. The material was blended in a Patterson-Kelley V-blender, and after homogenization, six samples taken at random were subject to particle size analyses using a Horiba LA-950 Light Scattering Particle Analyzer. For each sample, the particle size mean and range, and the percentage of particles below a certain size were calculated (Table 1). On average, the particle size of CRM AOCS 0224-A was 113.2 $\pm$ 6.6 µm, and 99% of the particles (i.e. D99) were smaller than 334.8 $\pm$ 33.6 µm.

Table 1. Results Particle Size Analyses of AOCS CRM 0224-A								
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Sample 5 (µm)**	**Sample 6 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	105.9	117.0	105.9	115.1	112.7	122.7	**113.2**	**6.6**
Range	0.77-344	0.88-452	0.77-344	0.88-452	0.88-394	0.88-452	N/A[(a)]	N/A[(a)]
D5[(b)]	8.1	8.4	8.2	8.2	8.4	8.3	8.3	0.1
D10[(b)]	14.4	14.5	14.5	14.4	14.4	14.3	14.4	0.1
D50[(b)]	91.5	102.0	92.7	92.7	97.3	104.2	96.7	5.4
D90[(b)]	225.9	249.0	224.1	255.6	241.7	265.8	243.7	16.5
D99[(b)]	298.4	341.8	296.9	368.5	327.6	375.6	**334.8**	**33.6**

[(a)] N/A = not applicable

[(b)] D5, D10, D50, D90, and D99 indicate that 5%, 10%, 50%, 90%, or 99% of the particles, respectively, are smaller than size given in table

The particle size distribution for each of the samples analyzed is presented in Figure 1 with the x-axis showing discrete size bins up to 517.2 µm. 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.



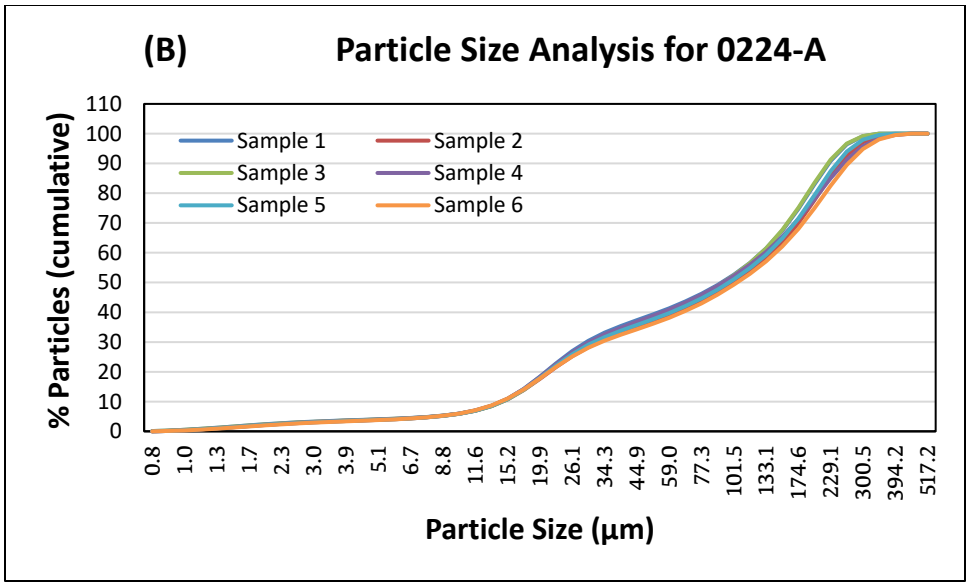


Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes.

Bulk, non-modified maize seed powder for the production of CRM AOCS 0224-A was delivered to AOCS. The powder 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).

Trait Verification

After the bulk material was packaged, the random number generator function of Microsoft® Excel was used to select samples. Ten randomly selected AOCS CRM 0224-A samples (19, 95, 132, 185, 211, 256, 288, 336, 388, and 424) were sent to Eurofins GeneScan GmbH, Freiburg, Germany (an ISO 17025:2005 accredited laboratory) for CRM homogeneity testing and verification. The absence of Event MZIR60 in non-modified maize material was assessed using event-specific quantitative and qualitative PCR. Based on quantitative PCR, MZIR260-specific DNA was below the practical Limit of Quantification (pLOQ) of 1% (expressed as mass/mass). Based on qualitative PCR, the level of MZIR260 maize DNA was below the validated method-specific Limit of Detection

(LOD) of 0.01% (expressed as copy/copy) as the mean Cq values of the CRM samples were higher than that for the LOD control sample (Table 2).

Table 2. Qualitative and quantitative results for the homogeneity and verification of CRM AOCS 0224-A non-modified maize as tested by Eurofins-GeneScan with a MZIR260-specific, quantitative and qualitative PCR method.

Sample	Event MZIR260 Presence (Quantitative Method)	Mean Cq Values[a] (Qualitative Method)
AOCS 0224-A 19	< 1.0 %	37.22 ± 0.62
AOCS 0224-A 95	< 1.0 %	38.46 ± 0.26
AOCS 0224-A 132	< 1.0 %	37.82 ± 0.27
AOCS 0224-A 185	< 1.0 %	37.66 ± 0.80
AOCS 0224-A 211	< 1.0 %	38.17 ± 0.81
AOCS 0224-A 256	< 1.0 %	37.52 ± 1.12
AOCS 0224-A 288	< 1.0 %	37.31 ± 0.45
AOCS 0224-A 336	< 1.0 %	37.00 ± 0.66
AOCS 0224-A 388	< 1.0 %	37.19 ± 0.67
AOCS 0224-A 424	< 1.0 %	37.83 ± 0.68

[a] The mean Cq values for each CRM samples were higher than the mean Cq value (36.62 ± 0.75) of the method-specific LOD control samples. Mean and standard deviation are reported from nine replicates of LOD control samples and up to six replicates of each CRM samples (six replicate reactions were used per CRM samples, but only some replicates showed amplification).

Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce AOCS 0224-A was assessed by Syngenta Seeds, LLC. A total of 4800 maize seeds (16 pools of 300 seeds) were tested by qualitative PCR to confirm the absence of MZIR260 maize, genetically modified (GM) impurities from commercial corn events where testing methodology is available, and regulated corn events under development at Syngenta. None of the 4800 seeds tested positive for the presence of any of the GM events including MZIR260.

Purity estimation was calculated based on the percentage of GM impurities using SeedCalc8 (Remund *et al*., 2008). The GM impurity in the sample was 0% when 4800 seeds were tested.

The measurement uncertainty is the expanded uncertainty using the value of the upper bound of impurity at 0.6 g/kg. The standard uncertainty can be obtained by dividing the expanded uncertainty by 2√3 (rectangular distribution).

The standard uncertainty for this CRM is 0.2 g/kg.

Homogeneity

The homogeneity of AOCS 0224-A is related to the purity of the seeds. A total of 4800 seeds were tested and all were negative for the MZIR260 maize. Based on the sample GM impurity of 0%, as determined using SeedCalc8, the batch was expected to be homogenous.

In addition, the homogeneity of the non-modified maize AOCS CRM 0224-A was confirmed using 10 random vials. The vials were sent to Eurofins-GeneScan GmbH, Freiburg, Germany (an ISO 17025:2005 accredited laboratory) for event-specific qualitative and quantitative real-time PCR analysis. For each AOCS CRM 0224-A sample, two independent DNA extractions and quantifications were performed using a test portion of 1 gram. Extracted DNA was checked for integrity by gel-electrophoresis and quantified prior to using it in real-time PCR. For each of the DNA extracts, all PCR reactions were done in triplicate.

The DNA extracts were analyzed by a qualitative and quantitative MZIR260-specific PCR method (GS-P-07.145). Based on quantitative PCR, MZIR260-specific DNA was below the practical Limit of Quantification (pLOQ) of 1% (expressed as mass/mass). Based on qualitative PCR, the level of MZIR260 maize DNA was below the validated method-specific Limit of Detection (LOD) of 0.01% (expressed as copy/copy) as the mean Cq values of the CRM samples were higher than that for the LOD control sample (Table 2). These results are in agreement with the Certified Value and Measurement Uncertainty section above.

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 GmbH, Engesserstraße 4, D-79108 Freiburg, Germany; Telephone: +49 761 6400 4022 Fax: +49 761 6400 4011; https://www.eurofinsus.de/food-analysis/

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

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