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Certified Reference Materials

AOCS 0224-B

Report of the certification process for

MZIR260

Maize Certified Reference Materials

First Batch

OECD Unique Identifier SYN-ØØ26Ø-3

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Abstract

This report describes the preparation and certification of the maize Certified Reference Material (CRM) AOCS CRM 0224-B produced by AOCS Technical Services in 2024. The CRM have been prepared according to ISO 17034:2016 and are intended to serve as control material for third party testing of maize for transformation events. MZIR260 maize seed was provided by Syngenta Seeds, LLC. The MZIR260 maize powder was prepared by grinding the bulk seed at AVEKA, Inc., Woodbury, MN (an ISO 9001:2015 accredited facility). The MZIR260 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 presence and homogeneity of MZIR260 in maize was verified using event-specific, quantitative PCR analysis at Eurofins Genescan, Freiburg, Germany (an ISO 17025:2005 accredited laboratory). The certified mass value of MZIR260 in MZIR260 maize was based on the purity of the bulk seed material and with 95% confidence, the true value is ≥ 914 g/kg. AOCS CRM 0224-B 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 at, 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
Cycle threshold (Cq)	Number of PCR cycles required for the fluorescent signal to cross a threshold that exceeds background level
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
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
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 quantified
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
Trait : MZIR260	Maize trait protects against certain lepidopteran insect pests

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, AOCS CRM 0224-B 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 hemizygous IJ7010/AX5797(MZIR260) maize hybrid (i.e. MZIR260 maize) used in the preparation of AOCS CRM 0224-B resulted from the cross of female non-transgenic line IJ7010 and male line AX5707(MZIR260). Syngenta Seeds, LLC delivered approximately 5 kg MZIR260 maize seed to AVEKA, Inc. Woodbury, Minnesota (an ISO 9001:2015 accredited facility).

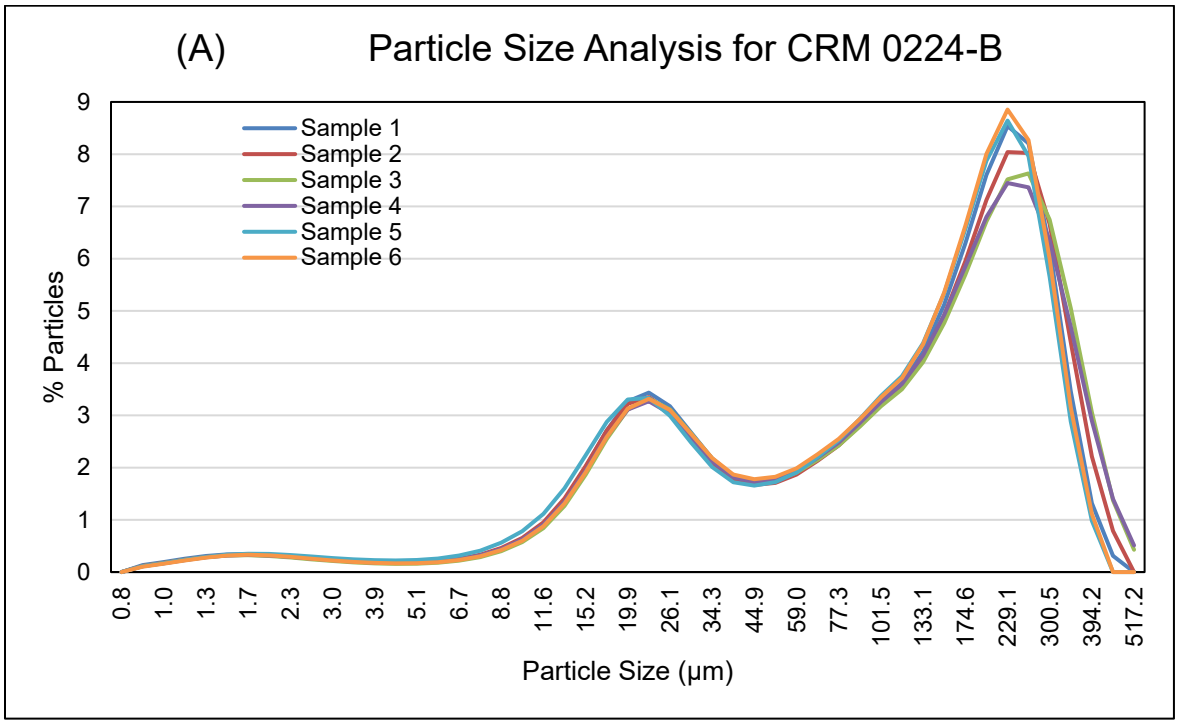
Bulk seed was milled at AVEKA, Inc. in a Fitzmill cryogenic hammermill using first a 510 µm screen. The material was blended in a Patterson-Kelley V-blender, and after homogenization, six samples taken at random were subject to particle size analyses using a Horiba LA-950 Light Scattering Particle Analyzer. For each sample, the particle size mean and range, and the percentage of particles below a given size was calculated (Table 1). On average, the particle size of CRM AOCS 0224-B was 134.6 $\pm$ 6.6 µm, and 99% of the particles (i.e. D99) were smaller than 384.5 $\pm$ 37.8 µm.

Table 1. Results of Particle Size Analyses of AOCS CRM 0224-B								
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Sample 5 (µm)**	**Sample 6 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	130.8	136.6	142.9	140.8	126.1	130.1	**134.6**	**6.6**
Range	0.88-452	0.88-452	0.88-517	0.88-517	0.88-394	0.88-394	N/A[(a)]	N/A[(a)]
D5[(b)]	9.5	9.6	10.4	9.9	8.4	10.2	9.7	0.7
D10[(b)]	15.5	15.4	16.2	15.7	14.3	15.9	15.5	0.6
D50[(b)]	121.1	125.1	128.6	125.7	117.2	122.7	123.4	4.0
D90[(b)]	270.0	285.1	300.0	297.3	260.2	264.0	279.4	17.2
D99[(b)]	367.2	389.2	426.7	430.6	343.9	349.0	**384.5**	**37.8**

[(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 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.



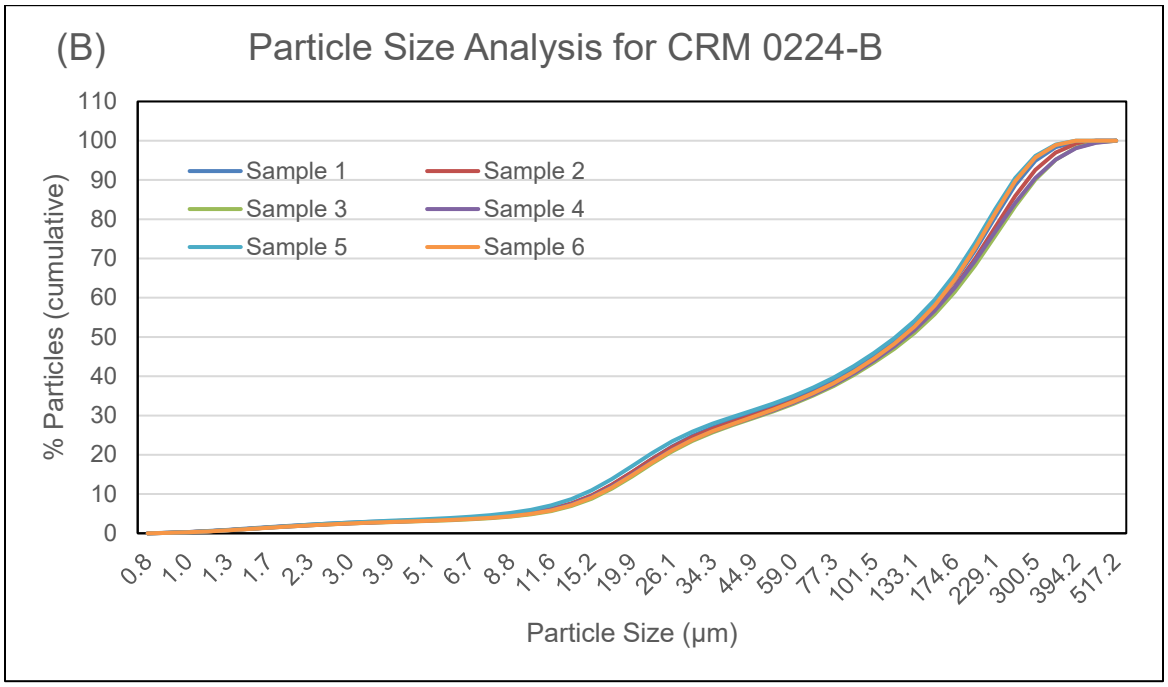


Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes.

Bulk maize powder was delivered to AOCS for the production of CRM 0224-B. The powder was then packaged in 27-mL glass headspace vials and sealed under a nitrogen gas environment at Illinois Crop Improvement Association (an ISO 17025:2017 accredited facility).

Trait Verification to Certify Presence of MZIR260

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-B samples (24, 88, 122, 165, 241, 285, 322, 340, 396, and 463) were sent to Eurofins GeneScan GmbH, Freiburg, Germany (an ISO 17025:2005 accredited laboratory) for event-specific PCR analysis to screen for MZIR260 presence in the samples. These results are presented in Table 2. This data confirm the presence of MZIR260 after the packaging of AOCS CRM 0224-B.

Table 2. Results for the verification of AOCS CRM 0224-B MZIR260 as tested by Eurofins GeneScan GmbH with a MZIR260 event-specific PCR method	
Sample Number	**MZIR260 Presence**
AOCS 0224-B 24	Positive
AOCS 0224-B 88	Positive
AOCS 0224-B 122	Positive
AOCS 0224-B 165	Positive
AOCS 0224-B 241	Positive
AOCS 0224-B 285	Positive
AOCS 0224-B 322	Positive
AOCS 0224-B 340	Positive
AOCS 0224-B 396	Positive
AOCS 0224-B 463	Positive

Certified Value and Measurement Uncertainty

The genetic purity based on the presence of MZIR260 in MZIR260 maize was assessed by Syngenta Crop Protection, LLC. A total of 398 MZIR260 maize seeds were evaluated by qualitative MZIR260-specific, real-time PCR. The results showed that 386 of the 398 seeds tested (96.98%) were positive for the presence of MZIR260.

Purity estimation was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponds to the lower bound of true % purity. The % purity in the sample was 96.98% when 398 seeds were tested. Using a 95% confidence level, the true % purity of the MZIR260 seed lot was 91.4%. Consequently, with 95% confidence, the true value is ≥ 914 g/kg.

The Measurement Uncertainty was based on the lower bound of the true % purity. The measurement uncertainty (U_{CRM}) is the expanded uncertainty with a coverage factor of 2 and a confidence level of 95%. It is obtained by combining the uncertainties from the purity assessment ($u_{char,rel}$), the homogeneity assessment ($u_{bb,rel}$), the transport stability assessment ($u_{sts,rel}$) and the long-term stability assessment ($u_{lts,rel}$):

$$u_{CRM,rel} = \sqrt{{u_{char,rel}}^2 + {u_{bb,rel}}^2 + {u_{sts,rel}}^2 + {u_{lts,rel}}^2}$$

$$U_{CRM} = 2 \times u_{CRM,rel} \times 1000\ g/kg$$

The expanded measurement uncertainty for AOCS 0224-B is -56 g/kg, +30 g/kg.

Homogeneity

The homogeneity of AOCS CRM 0224-B, MZIR260 maize is related to the purity of the seeds. 386 out of 398 seeds tested positive for the MZIR260 maize event by event-specific PCR. Based on the sample purity of 96.98%, as determined by SeedCalc8, the batch is expected to be homogenous.

To further confirm homogeneity 10 vials (randomly selected as described above) were also assessed after bottling of AOCS CRM 0224-B using a MZIR260 specific, quantitative real-time PCR method (). The ten samples of AOCS CRM 0224-B maize were analyzed, and for each sample, two independent DNA extractions and quantifications were performed at Eurofins Genescan GmbH (an ISO 17025:2005 accredited laboratory) using a test portion of 1 g. 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 cycle threshold (Ct) values for an endogenous *adh1* maize gene and for event MZIR260 were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between event MZIR260 copy number and *adh1* copy number (MZIR260 cp#/*adh1* cp#) was calculated and used to estimate within-unit relative stand-

ard 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 3. The within-unit relative standard deviation (RSD_w) and the between-unit relative standard deviation (RSD_b) for vials of AOCS CRM 0224-B.

CRM	RSD_w [%]	RSD_b [%]	$u^*_{bu\ rel}$ [%]
AOCS 0224-B	4.6	n.c.[1]	2.2

[1]n.c: RSD_b cannot be calculated as $MS_{between} < MS_{within}$. In this situation, maximum hidden inhomogeneity ($u^*_{bu\ rel}$) is provided as an alternative

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 ≤ 20%. Based on the quantitative real-time PCR analyses conducted, it was concluded that AOCS CRM 0224-B is homogenous (Table 3). These results are in agreement with homogeneity results from qualitative real-time PCR analyses and from the purity estimate for MZIR260 material calculated in 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.

The effect of temperature and time are investigated.
A transport (short-term) stability study is conducted to assess the stability of maize CRM during transport. The temperature and time conditions in the study cover the typical conditions and not so rare situations. The outcome of the study is considered transferable to other CRMs of similar property. Samples were subject to 3 different temperatures (4ºC (fridge), 25 ºC (ambient), 60 ºC(oven)) for 4 different durations (0, 1, 2, and 4 weeks). The study concluded that samples are stable at 4 ºC (fridge) and 25 ºC (ambient) for 4 weeks. The estimated uncertainty contribution from transport (short-term) stability is 1.0%.
A long-term stability study is conducted to assess the stability of maize CRM during storage. Samples are stored at 25 ºC (ambient) and the stability of the sample is monitored as long as the samples is available. The storage temperate studied is 25 ºC and the length of time to be studied is 10 years. The outcome of the study is considered transferable to other CRMs of similar property. In the initial 1-year stability study, samples were subject the storage condition for 4 different durations (0, 1, 3, 6 and 12 months). The study concluded that samples are stable at 25 ºC (ambient) for 12 months. The estimated uncertainty contribution from long-term stability is 0.42%.

CRM stability over time will be analyzed by repeating the homogeneity study described above at a chosen shelf life of approximately every 24 months. The 24-month shelf life of CRM is chosen because the influence of analytical variation can be reduced by increasing the length of the stability study (Linsinger *et al*., 2001).

The initial ratio between the number of copies of the GM event and the number of copies of the endogenous reference gene from the homogeneity study will establish the base line for the stability study. The ratio at each 24-month interval will be compared to the ratio established in the homogeneity study. The CRM will be determined to be stable if the variability of the ratios, determined as relative standard deviation (RSD) is ≤ 20%.

Stability of these CRMs has been listed as 2 years 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; 2045 Wooddale Drive. Woodbury, MN 55125; Telephone : +1 651 730 1729; https://www.aveka.com/

Eurofins-GeneScan GmbH, Engresserstraße 4, D-79108 Freiburg, Germany Telephone: +49 761 6400 4011 Online: 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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