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Certified Reference Materials

AOCS 0407-B

Report of the certification process for

GA21

Maize Certified Reference Materials

First Batch

OECD Unique Identifier MON-ØØØ21-9

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Table of Contents

Abstract

This report describes the preparation and certification of the maize AOCS CRM 0407-B produced by AOCS Technical Services in 2007. The CRMs have been prepared according to ISO Guides 30, 31, 34 and 35 and are intended to serve as control material for third party testing of maize for transformation events. GA21 maize seed was provided by Syngenta Crop Protection, LLC. The GA21 maize powder was prepared by grinding the bulk seed according to maize processing protocols at Texas A&M University and was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association. The presence of GA21 in maize was verified using event-specific, qualitative PCR analysis at Eurofins Genescan, Metairie, LA (an ISO 17025:2005 accredited laboratory). AOCS CRM 0407-B is available in 27-mL glass headspace vials. The certified mass value of GA21 in GA21 maize was based on the purity of the material and with 95% confidence, it is higher than 916 g/kg. Homogeneity testing was performed at Eurofins GeneScan using qualitative real-time PCR prior to bottling, and quantitative real-time PCR after the AOCS CRM 0407-B was bottled. AOCS CRM 0407-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 Monique Nesbitt, Syngenta Crop Protection, LLC, for offering AOCS the opportunity to manufacture and distribute these products; to Richard Clough, Texas A&M University for processing the samples; to Sandra Harrison and Charlie Drennan at Illinois Crop Improvement Association for packaging the samples; and to Frank Spiegelhalter, Greg Ditta, and E. Pearce Smith, 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 (Ct)	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
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: GA21	Tolerance to herbicides containing glyphosate

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 0407-B was manufactured from maize seed according to ISO Guides 30, 31, 34 and 35 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 GA21 maize used in the preparation of AOCS CRM 0407-B resulted from the cross of female non-transgenic NP2391 and male NP2673 (GA21), and Syngenta Crop Protection, LLC delivered 20 kg of GA21 maize seed to AOCS. The GA21 maize was ground at the Texas A&M Food Protein Research and Development Center using a hammer mill fitted with a 1/8 inch screen. The maize flour was then packaged in 27-mL glass headspace vials and sealed under a nitrogen gas environment at Illinois Crop Improvement Association.

Once packaged, four randomly selected samples were subject to particle size analysis. Particle size analysis was conducted at AVEKA, Woodbury, Minnesota, (an ISO 9001:2015 accredited laboratory) using a Horiba LA-950 particle size analyzer. For each sample, the particle size mean and range, and the percentage of particles below a given size was calculated. On average, the particle size of AOCS CRM 0407-B was 254.99 +

3.16 µm, and 90% of the particles (i.e. D90) were smaller than 515.78 + 12.28 µm (Table 1)

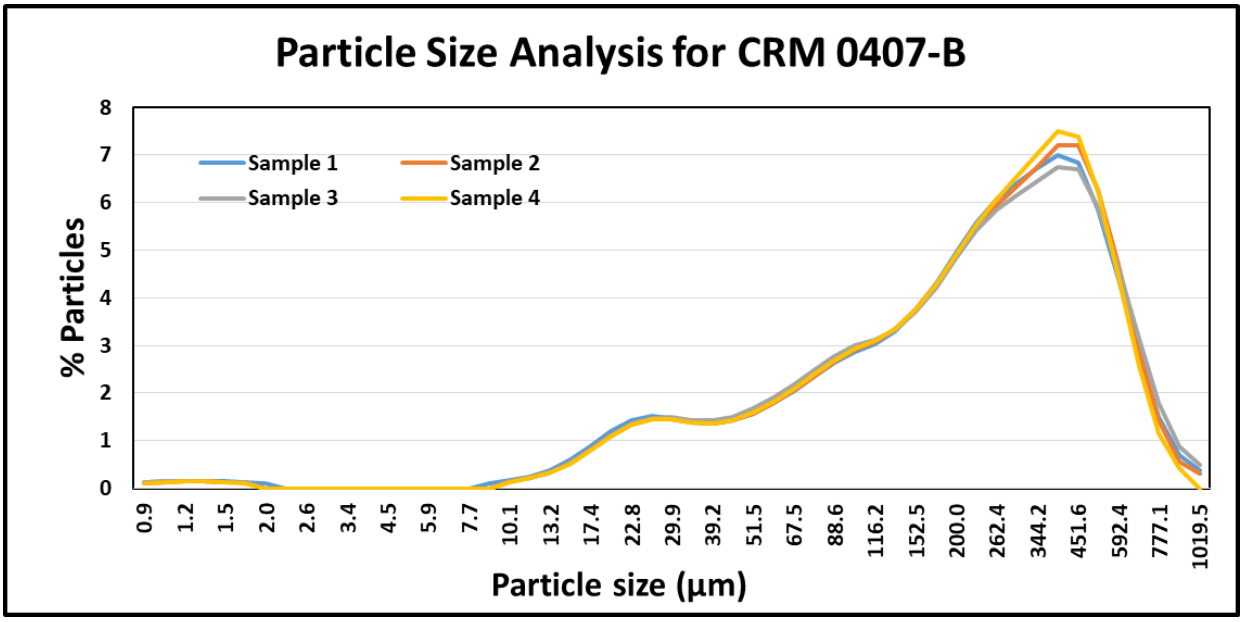
Table 1. Results of Particle Size Analyses of AOCS CRM 0407-B

	Sample 1 (µm)	Sample 2 (µm)	Sample 3 (µm)	Sample 4 (µm)	Average (µm)	Standard Deviation (µm)
Mean	253.59	257.04	258.09	251.23	**254.99**	**3.16**
Range	0.90-1020	0.90-1020	0.90-1020	0.90-890	N/A[(a)]	N/A
D10 [(b)]	33.06	35.81	35.16	36.15	35.05	1.39
D50 [(b)]	221.78	226.83	221.85	225.40	223.96	2.55
D90 [(b)]	514.30	516.06	531.36	501.39	**515.78**	**12.28**

[(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 discrete size bins up to 3000 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.



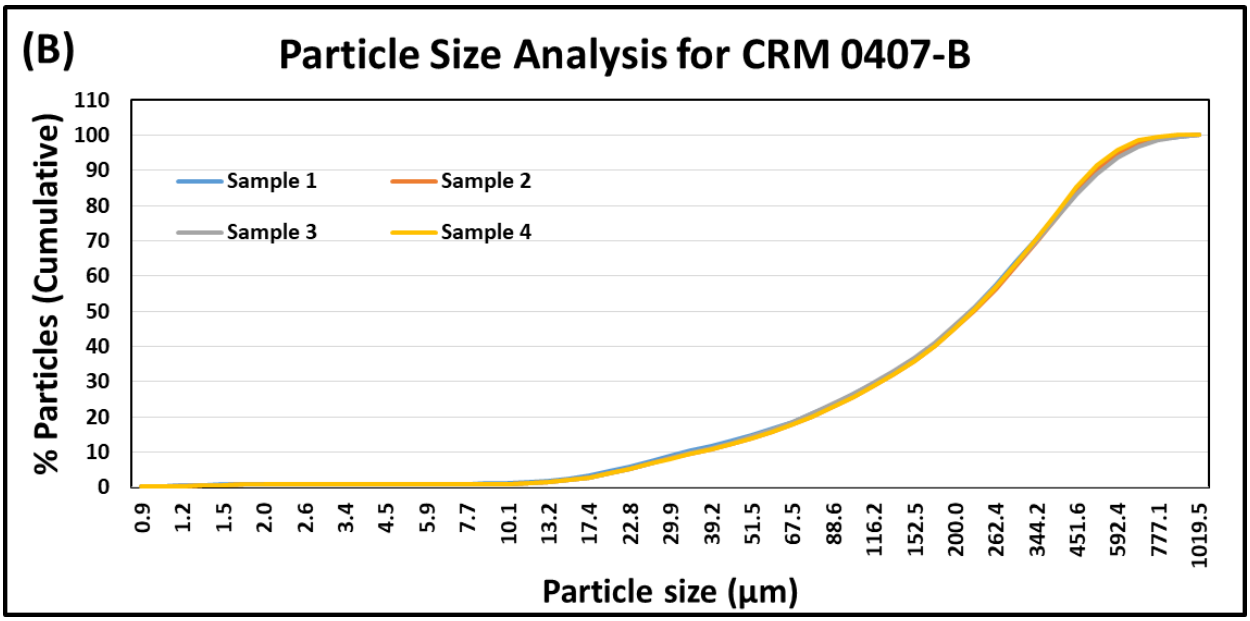


Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes.

Trait Verification

After the bulk material was packaged, AOCS used the random number generator function of Microsoft Excel to select samples for verification of trait presence, homogeneity, and to rule out degradation during packaging. A total of ten (10) randomly selected AOCS CRM 0407-B samples (29, 47, 67, 115, 159, 217, 220, 402, 596 and 712) were sent to

Eurofins Genescan, Metairie, LA for event-specific, qualitative PCR analysis to screen for GA21 presence in the samples. These results are presented in Table 2. These data confirm the presence of GA21 after the packaging of AOCS CRM 0407-B.

Table 2. Results for the verification of AOCS CRM 0407-B GA21 as tested by Eurofins GeneScan with a GA21 event-specific, qualitative PCR method

Sample Number	GA21 Presence
AOCS 0407-B 29	Positive
AOCS 0407-B 47	Positive
AOCS 0407-B 67	Positive
AOCS 0407-B 115	Positive
AOCS 0407-B 159	Positive
AOCS 0407-B 217	Positive
AOCS 0407-B 220	Positive
AOCS 0407-B 402	Positive
AOCS 0407-B 596	Positive
AOCS 0407-B 712	Positive

Certified Value and Measurement Uncertainty

The genetic purity based on the presence of GA21 in GA21 maize was assessed by Syngenta Crop Protection, LLC. A total of 352 GA21 maize seeds were evaluated by qualitative GA21-specific, real time PCR. The results showed that 352 of the 352 seeds tested (100%) were positive for the presence of GA21.

The statistical purity of GA21 maize was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponds to the lower bound of true % purity. Using a 95% confidence level,

the true % purity of GA21 maize is at least 91.6 %Consequently, with 95% confidence, the true value is $\geq$ 916 g/kg.

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 ($u_{lts,rel}$):

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

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

Consequently, the expanded measurement uncertainty for this CRM is -84 g/kg.

Homogeneity Testing

The material used for the production of AOCS CRM 0407-B, GA21 maize, is 100% pure (see Certified Value and Measurement Uncertainty section) and is expected to be homogenous. However, to further confirm homogeneity, the ground material was analytically assessed before and after bottling using real-time PCR.

Primary samples were taken from randomly selected areas and depths to form a 3-kg composite sample in accordance with the International Seed Testing Association's (ISTA) Seed Science and Technology Rules for batches up to 500 kg. Ten (10) working samples of 10 g each were prepared from the composite sample and sent to Eurofins GeneScan, Metairie, LA (an ISO 17025:2005 accredited laboratory) for event-specific, qualitative PCR analysis. The analyses performed by Eurofins Genescan, Metairie, LA were used to assess the trait presence and homogeneity of the seed lot. A summary of the qualitative PCR results for the GA21 homogeneity samples is presented in Table 3.

Table 3. Homogeneity testing prior to bottling using GA21-specific qualitative real-time PCR on the GA21 bulk material provided by Syngenta Crop Protection, LLC	
Sample Number	**GA21 Presence**
Homogeneity Sample 1	Positive
Homogeneity Sample 2	Positive
Homogeneity Sample 3	Positive
Homogeneity Sample 4	Positive
Homogeneity Sample 5	Positive
Homogeneity Sample 6	Positive
Homogeneity Sample 7	Positive
Homogeneity Sample 8	Positive
Homogeneity Sample 9	Positive
Homogeneity Sample 10	Positive

In addition, homogeneity was also assessed after bottling of AOCS CRM 0407-B using a GA21 specific, quantitative real-time PCR method (http://gmo-crl.jrc.ec.europa.eu/gmomethods/docs/GA21_Validation_Report+Protocol[1].pdf). A total of 10 samples of AOCS CRM 0407-B maize were analyzed, and for each sample, 2 independent DNA extractions and quantifications were performed at Eurofins Genescan 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 GA21 were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between event GA21 copy number and *adh1* copy number (GA21 cp#/*adh1* cp#)

was calculated and used to estimate within- and between-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 4. The within-unit relative standard deviation (RSD_w) and the between-unit relative standard deviation (RSD_b) for vials of AOCS CRM 0411-D2.

CRM	RSD_w [%]	RSD_b [%]
AOCS 0407-B	8.6	4.1

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 0407-B is homogenous (Table 4). These results are in agreement with homogeneity results from qualitative real-time PCR analyses and from the purity estimate for material GA21 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 the 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 (4 °C (fridge), 25 °C (ambient), 60 °C (oven)) for 4 different durations (0, 1, 2, and 4 weeks). The study concluded that samples are stable at 4 °C (fridge) and 25 °C (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 °C (ambient) and the stability of the sample is monitored as long as the samples is available. The storage temperate studied is 25 °C 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 °C (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/

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: +1 504 846 2398; 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/

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