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Certified Reference Materials

AOCS 1012-A2

Report of the certification process for

Non-modified

Cotton Certified Reference Materials

Second Batch

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Abstract

This report describes the preparation and certification of the cotton CRM AOCS 1012-A2 produced by AOCS Technical Services in 2020. The CRMs have been prepared according to ISO 17034:2016 and are intended to serve as control material for third party testing of cotton for transformation events. Non-modified cotton powder 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 cotton 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 1012-A2 was based on the absence of genetically modified impurities and with 95% confidence, is lower than 1 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 1012-A2 was bottled. Homogeneity results indicated that CRM AOCS 1012-A2 is homogeneous and were used to verify the absence of Event COT102 in this CRM. CRM AOCS 1012-A2 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, 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	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
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
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 regulatory requirements for GMO determination, CRM AOCS 1012-A2 was manufactured from cotton 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

Non-modified cotton was used for the production of CRM AOCS 1012-A2. Non-modified cotton seed 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 Fitzpatrick 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 given size was calculated (Table 1). On average, the particle size of CRM AOCS 1012-A2 was 155.47 ± 4.01 µm, and 90% of the particles (i.e. D90) were smaller than 331.15 ± 7.62 µm.

Table 1. Results of Particle Size Analyses of CRM AOCS 1012-A2 Conducted by AVEKA								
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Sample 5 (µm)**	**Sample 6 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	154.91	158.75	161.27	149.94	153.41	154.52	155.47	4.01
Range	0.58-517.20	0.58-592.39	0.58-678.50	0.58-517.20	0.58-592.39	0.51-592.39	N/A[(a)]	N/A
D10 [(b)]	17.08	17.19	17.45	16.73	16.38	15.42	16.71	0.73
D50 [(b)]	138.83	142.32	141.05	132.17	134.42	136.97	137.63	3.89
D90 [(b)]	328.30	335.36	343.47	320.81	329.20	329.77	331.15	7.62

(a) N/A = not applicable

(b) D10, D50 and D90 indicate that 10%, 50% or 90% of the particles, respectively, are smaller than largest 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 680 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 the particles were ≤ 678.5 µm.

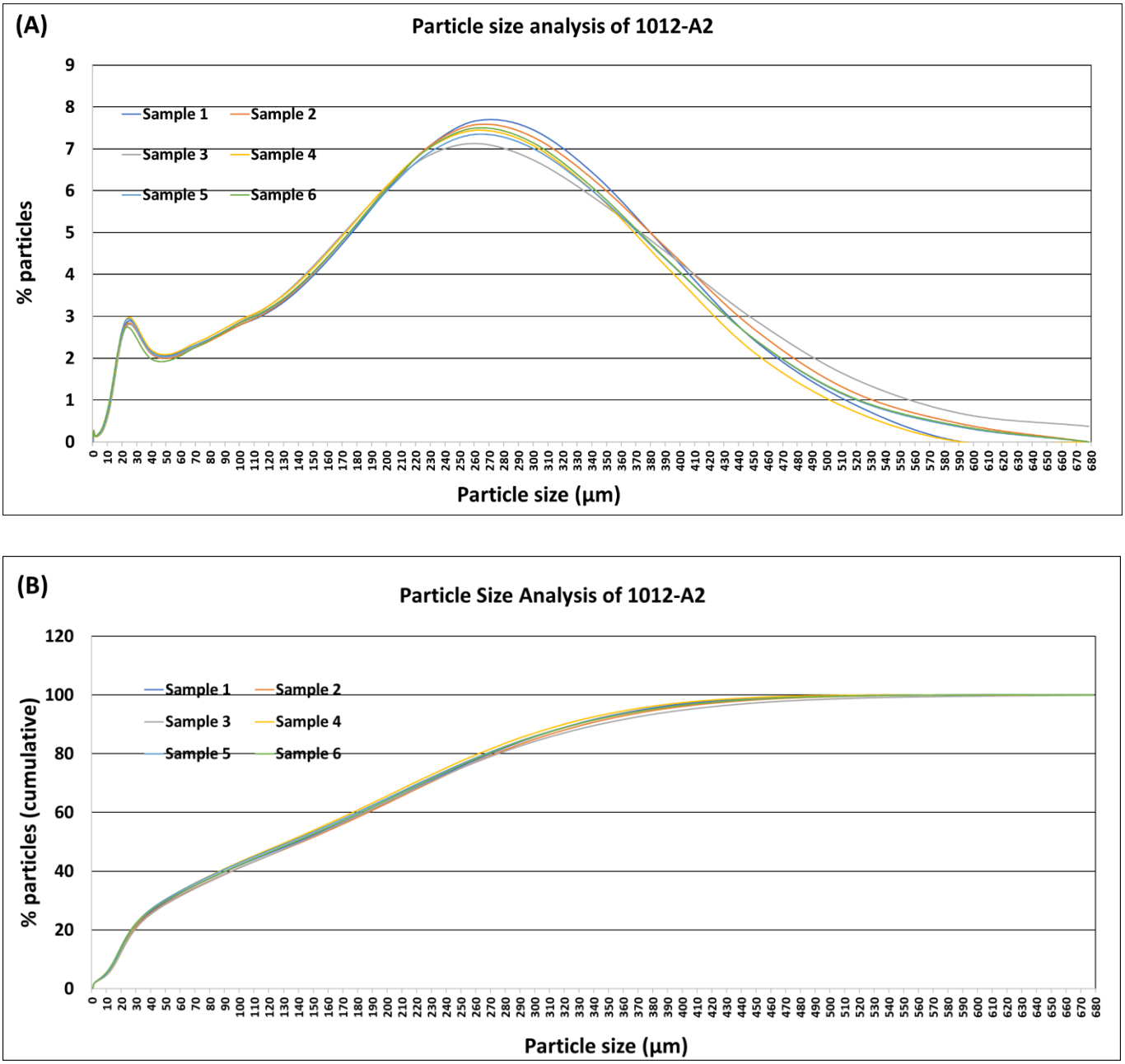

Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes

Bulk, non-modified cotton seed powder for the production of CRM AOCS 1012-A2 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 CRM AOCS 1012-A2 was assessed by Syngenta Crop Protection, LLC. A total of 3000 seeds (6 pools of 500 seeds/pool) of non-modified cotton were evaluated by real-time PCR at Syngenta Crop Protection, LLC. None of the non-modified cotton seed pools tested positive for COT102 cotton.

Purity estimation in non-modified cotton was calculated using SeedCalc8 (Remund *et al*., 2008). The % impurity in the sample was 0% when 3000 seeds were tested. Based on the upper bound of true % purity and with 95% confidence, true certified value is < 1 g/kg.

The measurement uncertainty (U_{CRM}) is the expanded uncertainty with a coverage factor of 1.65 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 ($u_{lts,rel}$):

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

$$U_{CRM} = 1.65 \times u_{CRM,rel} \times 1000\ g/kg$$

The expanded measurement uncertainty for this CRM is 0.5 g/kg.

Homogeneity Testing

The non-modified cotton material used for the production of CRM AOCS 1012-A2 contains 0% impurities and is expected to be homogenous. After the non-modified cotton seed was ground and bottled as described above, ten samples of CRM AOCS 1012-A2 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 1012-A2 was based on the quantification of the endogenous *sah7* cotton gene using real-time PCR method (https://gmo-

crl.jrc.ec.europa.eu/summaries/EURL-VL-05-16-VM.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 event COT102 in CRM AOCS 1012-A2 was assessed using a COT102-specific, quantitative, real-time PCR method (https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-05-16-VM.pdf)

The cycle threshold (Ct) values for the endogenous *sah7* cotton gene and for Event COT102 were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between Event COT102 copy number and *sah7* copy number (COT102 cp#/*sah7* 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 CRM AOCS 1012-A2.

CRM	RSD_w [%]	RSD_b [%]
AOCS 1012-A2	Below LOQ	Below LOQ

The CRM will be determined to be homogeneous if the within-unit relative standard deviation (RSD_w) and between-unit relative standard deviation (RSD_b) are both ≤ 20%. Based on the quantitative real-time PCR analyses conducted, it was concluded that CRM AOCS 1012-A2 is homogenous (Table 2) because it did not contain traces of COT102 above the LOQ of the applied real-time PCR method. These results are in agreement with the Certified Value and Measurement Uncertainty section above.

Trait Verification

The absence of Event COT102 in the non-modified cotton material was assessed in the same ten CRM AOCS 1012-A2 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. None of the samples contained traces above the limit of detection of the COT102-specific method (https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-05-16-VM.pdf) and therefore were negative for Event COT102.

Table 3. Qualitative results for the verification of CRM AOCS 1012-A2 as tested by Eurofins GeneScan with a COT102 -specific, quantitative PCR analysis

Sample	Event COT102 Presence
AOCS 1012-A2 11	Negative
AOCS 1012-A2 33	Negative
AOCS 1012-A2 60	Negative
AOCS 1012-A2 97	Negative
AOCS 1012-A2 187	Negative
AOCS 1012-A2 254	Negative
AOCS 1012-A2 335	Negative
AOCS 1012-A2 379	Negative

AOCS 1012-A2 456	Negative
AOCS 1012-A2 528	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.

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 ℃ (fridge), 25 ℃ (ambient), 60 ℃ (oven)) for 4 different durations (0, 1, 2, and 4 weeks). The study concluded that samples are stable at 4 ℃ (fridge) and 25 ℃ (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 ℃ (ambient) and the stability of the sample is monitored as long as the samples is available. The storage temperate studied is 25 ℃ 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 ℃ (ambient) for 12 months. The estimated uncertainty contribution from long-term stability is 0.42%.

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 listed on the certificate. The stability of the powder material will be re-evaluated at the scheduled time of shelf life, approximately 12-month intervals. 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: +1 504 297 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/

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