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Certified Reference Materials

AOCS 0117-A and 0117-E

Report of the certification process for

LBFLFK

Canola Certified Reference Materials

First Batch

OECD unique identifier BPS-BFLFK-2

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ISO 17034:2016
A2LA Certificate 3438.01

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Table of Contents

Abstract

This report describes the preparation and certification of the canola certified reference materials (CRMs) AOCS 0117-A and 0117-E produced by AOCS Technical Services in 2018. The CRMs have been prepared according to ISO 17034:2016 and are intended to serve as control material for third party testing of canola for genetically modified (GM) events. The presence of LBFLFK in the canola was verified using event-specific, qualitative PCR analysis by Eurofins-GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory). AOCS 0117-A and 0117-E are available in 1-gram amount contained in 6 mL glass headspace vials. The canola seed was provided by BASF Plant Science, L.P. The canola LBFLFK powder and the non-GM canola powder were prepared by grinding the bulk source according to canola processing protocols by Texas A&M University and were then packed under a nitrogen gas environment at Protoform, Inc. The powder sample 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 Huihua Fu and Connie Martin, BASF Plant Science, L.P., for offering AOCS the opportunity to manufacture and distribute these products; to Richard Clough, Texas A&M University for processing the samples; to Jeff Gazzara, Protoform, Inc. for mixing and bottling the samples; and to Frank Spiegelhalter, Greg Ditta, E. Pearce Smith, and Daniel Thompson, Eurofins-GeneScan for event-specific, qualitative PCR analysis including the provision of information on running the analyses and interpreting the results.

Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	Crop variety with no history of modern biotechnology and is produced through plant-breeding techniques that rely on selecting and mating parent plants possessing promising traits and repeatedly selecting for superior performance among their offspring, also referred as non-GM crop.
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 exist in a sample can be reproducibly measured. It is typically expressed as the ratio of the number of modern biotechnology derived genomes to the number of crop genomes times 100 percent
EC	European Commission
Genome	The full set of genes and associated DNA characteristic of an organism
ISO	International Organisation for Standardisation
GMO	Organism that has had genetic sequences modified using molecular-level techniques
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 makes enough copies of 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 targeted DNA sequence in a sample can be reproducibly measured. It is typically expressed as the ratio of the number of transgenic genomes to the number of crop genomes times 100 percent.
Quantitative PCR	PCR methods that estimate the relative amount of target DNA sequence in a mixture of DNA molecules
LBFLFK	EPA + DHA canola, OECD unique identifier BPS-BFLFK-2, created in a single transformation experiment by introduction of a construct that confers the production of long-chain polyunsaturated fatty acids and an herbicide tolerance trait. LBFLFK contains two T-DNA inserts (‘Insert1’ and ‘Insert2’) that are integrated in two separate loci (Locus1 and Locus2) of the canola (*Brassica napus* L.) genome.

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 created from leaf, seed, or grain containing the new trait as well as a CRM created from the conventionally bred matrix. The European Commission (EC) has mandated that from 18 April 2004, a method for detecting a new event derived from modern biotechnology and Certified Reference Material must be available before the EC will consider authorizing acceptance of a new crop derived from modern biotechnology. 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, AOCS 0117-A and 0117-E were manufactured from canola according to ISO 17034:2016 and in accordance with EC No 1829/2003, EC No 641/2004and EC No 619/2011. These CRMs are available from AOCS.

Materials Processing

AOCS 0117-A and 0117-E have been prepared from non-modified (non-GM) and genetically modified (GM) canola seeds provided by BASF Plant Science, L.P. The materials were clean homozygous seeds. BASF performed seed purity and adventitious presence (AP) testing on the GM LBFLFK and non-GM counterpart parental variety seed lots prior to transferring the seed lots to AOCS. Before the materials were shipped for uniform processing, AOCS randomly choose 20 representative LBFLFK canola seeds and 20 representative non-GM canola seeds, placed them individually in new Ziploc bags, and crushed each seed with pliers or a hammer. Eurofins-Genescan New Orleans, LA (an ISO 17025 accredited laboratory) tested the seeds for event-specific, qualitative PCR analysis, for presence of the LBFLFK event to confirm the purity assessment conducted

by BASF. All 20 GM seeds tested positive for LBFLFK and all 20 non-GM seeds tested negative. Therefore, the BASF purity analyses was confirmed by AOCS.

AOCS sent the devitalized seeds to Texas A&M for an initial grinding. Texas A&M ground the seeds while taking appropriate steps to prevent cross-contamination of samples. Batches were ground using a hammer mill fitted with an 1/16-inch screen. All work at Texas A&M was conducted under an established protocol provided by AOCS that ensures temporal and spatial separation of GM and non-GM seed including extensive cleaning of the apparatus used for grinding. The GM and non-GM canola powders were processed separately. Cross-contamination with foreign DNA was avoided by using clean laboratory clothing and keeping GM and non-GM powders spatially separate to avoid cross-contamination by air. All contact surfaces were treated with a DNA degrading solution prior to exposure to the materials.

The GM and non-GM powders were homogenized separately by 0.5 h of mixing using a T2 C Turbula mixer at 46 rpm. Powders were sifted through a 500 µm sieve and coarse fractions that failed to pass were ground with a track mill taking care to avoid exposing the powders to high temperatures. The process was repeated until the majority (99%) of the GM and non-GM powders passed through a 500 µm sieve. Both powders were then homogenized again separately by 0.5 h of mixing. The GM and non-GM powders were then dried under vacuum.

All powders were aliquoted into 6 mL headspace vials at masses of approximately 1g. All vials were sealed under a nitrogen gas environment.

Particle Size Analysis

Particle size volume distributions for the homogenized GM and non-GM powders were measured by laser diffraction (Figure 1). It was concluded that the particle volume fractions of the homogenized powders confirmed enough physical homogeneity.

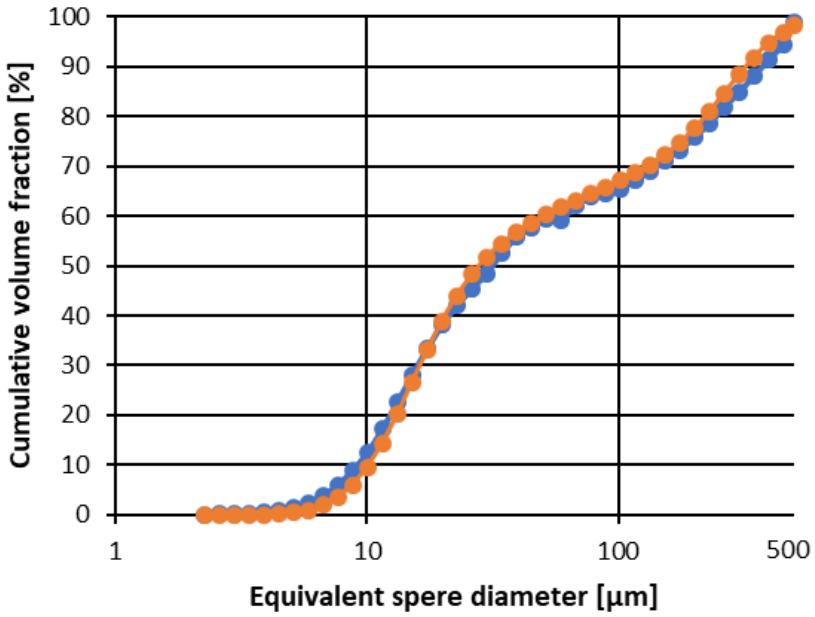

Figure 1: Cumulative particle volume fractions in the GM powder (□) and non-GM powder (□) analyzed by laser diffraction (N = 1, n = 3).

The AOCS 0117-A and 0117-E CRMs were prepared from the homogenized non-GM and GM powders that were solely from identity preserved homozygous canola seed lots and therefore sample genetic heterogeneity was not considered.

Trait Verification to Certify Presence of LBFLFK

The presence of LBFLFK in AOCS 0117-A and 0117-E was determined event-specific real-time PCR conducted by Eurofins-Genescan New Orleans, LA (an ISO 17025 accredited laboratory). DNA was extracted from 100 mg GM and non-GM powders by thermal lysis in the presence of Tris HCL, EDTA, SDS and β-mercaptoethanol, removal of contaminants by extraction with phenol and chloroform, precipitation of DNA using isopropanol and dissolution in TE-buffer. The yields of DNA were measured using the Quant-iT™ PicoGreen® dsDNA Assay kit. None of the samples analyzed for DNA integrity showed DNA degradation.

Results of the confirmation measurements are presented in Table 1.

Table 1: Confirmation measurements for presence of LBFLFK by event-specific real-time PCR conducted by Eurofins-Genescan New Orleans, LA (an ISO 17025 accredited laboratory)

CRM	LBFLFK mass fraction [g/kg]	*U* (*k* =1.65) [1)] [g/kg]
0117-A	0	-
0117-E	1000	4

1) The expanded uncertainty of the qPCR measurements.

Certified Value and Measurement Uncertainty

Certified values were calculated based on the genetic purity of the GM and non-GM seed lots. Event-specific real-time PCR (conducted by an ISO 17025 accredited laboratory) was used to confirm the genetic purity.

3000 out of 3000 seeds from the non-GM seed lot tested negative for the LBFLFK event. Purity estimation was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponds to the upper bound of true % purity. The % impurity in the sample was 0%, when 3000 seeds were tested. The Measurement Uncertainty of 1 g/kg was based on the upper bound of impurity. The standard uncertainty can be obtained by dividing the expanded uncertainty by $2\sqrt{3}$ (rectangular distribution). The standard uncertainty for AOCS 0117-A is 0.3 g/kg

349 out of 349 seeds from the GM seed lot tested positive for the LBFLFK event. 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 100%, when 349 seeds were tested. Using a 95% confidence level, the true % purity of the LBFLFK seed lot was at least 97.7%. Therefore, the certified value of the nominal 100% pure LBFLFK CRM was assigned as > 977 g/kg. The Measurement Uncertainty of -23 g/kg was based on the

lower bound of the true % purity and is the expanded uncertainty with a coverage factor of 1.65 and 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 ($u_{lts,rel}$):

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

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

Minimum sample intake

Confirmation and stability experiments were performed using either 100 mg or 200 mg samples. Sampling at this intake yields acceptable intermediate precision, as within-unit homogeneity does not contribute to analytical variation. Using sample sizes equal or above 200 mg guarantees the certified value within its stated uncertainty.

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 °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%.

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 6 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 still test for the presence/absence of the intended trait, the certificates will be extended.

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http://www.cera-gmc.org/?action=gm_crop_database

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