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ERM
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CERTIFICATION REPORT

The certification of different mass fractions of DAS-81910-7 in cotton powder Certified Reference Materials ERM®-BF440a, ERM®-BF440b, ERM®-BF440c, ERM®-BF440d and ERM®-BF440e

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Abstract

This report describes the production of a set of Certified Reference Materials (CRMs), ERM-BF440a, b, c, d and e, which are certified for their DAS-81910-7 cotton (unique identifier DAS-81910-7) event mass fractions. These materials were produced following ISO Guide 34:2009 and are certified in accordance with ISO Guide 35:2006.
Genetically modified (GM) cotton seeds of the DAS-81910-7 event and seeds from a non GM cotton variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM cotton seed powder were prepared gravimetrically.
The certified values were obtained from the gravimetric preparations, taking into account the water mass fractions of the two powder materials and the genetic purity with respect to the DAS-81910-7 cotton. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements were within the scope of accreditation to ISO/IEC 17025:2005).
The uncertainties of the certified values were estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties relating to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).
The materials are intended for the calibration or quality control of real-time PCR measurements to identify DAS-81910-7 cotton and quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs are available in glass bottles containing at least 1 g of dried cotton seed powder, sealed under an atmosphere of argon. The minimum amount of sample to be used for extraction of the DNA is 200 mg.

CERTIFICATION REPORT

The certification of different mass fractions of DAS-81910-7 in cotton powder

Certified Reference Materials ERM®-BF440a, ERM®-BF440b, ERM®-BF440c, ERM®-BF440d and ERM®-BF440e

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of a set of Certified Reference Materials (CRMs), ERM-BF440a, b, c, d and e, which are certified for their DAS-81910-7 cotton (unique identifier DAS-8191Ø-7) mass fractions. These materials were produced following ISO Guide 34:2009 [1] and are certified in accordance with ISO Guide 35:2006 [2].

Genetically modified (GM) cotton seeds of the DAS-81910-7 event and seeds from a non-GM cotton variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM cotton seed powder were prepared gravimetrically.

The certified values were obtained from the gravimetric preparations, taking into account the water mass fractions of the two powder materials and the genetic purity with respect to the DAS-81910-7 cotton. The certified values were confirmed by event-specific quantitative PCR as an independent verification method (measurements were within the scope of accreditation to ISO/IEC 17025:2005 [3]).

The uncertainties of the certified values were estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties relating to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).
The materials are intended for the calibration or quality control of quantitative PCR measurements to identify DAS-81910-7 cotton and quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs are available in glass bottles containing at least 1 g of dried cotton seed powder, sealed under an atmosphere of argon. The minimum amount of sample to be used for extraction of the DNA is 200 mg.

The following values were assigned:

	DAS-81910-7 cotton mass fraction [1)]	
	Certified value [g/kg]	Uncertainty [g/kg] [5)]
ERM-BF440a	< 0.07 [2)]	-
ERM-BF440b	> 986 [3)]	-
ERM-BF440c	1.00 [4)]	0.08
ERM-BF440d	10.0 [4)]	0.8
ERM-BF440e	100 [4)]	7

1) Genetically modified cotton with the unique identifier DAS-8191Ø-7.

2) The certified reference material has been produced from conventional, non-modified cotton seeds. No contamination was detected in this material when using an event-specific quantitative polymerase chain reaction assay targeting the DAS-81910-7 cotton event. The limit of detection (LOD) was 0.07 g/kg. With 95 % confidence, the true DAS-81910-7 cotton mass fraction of the material is below 0.07 g/kg. The certified value is traceable to the International System of Units (SI).

3) This certified reference material was produced from genetically modified DAS-81910-7 cotton seeds. The certified value is based on the genetic purity of the cotton powder with regard to DAS-81910-7 cotton. In total 216 seeds were tested individually for the presence of the DAS-81910-7 cotton event. All seeds tested positive. With 95 % confidence, the true DAS-81910-7 cotton mass fraction of the material is above 986 g/kg. The certified value is traceable to the International System of Units (SI).

4) This certified value is based on the masses of dried genetically modified DAS-81910-7 cotton powder and dried non-modified cotton powder that were mixed, taking into account their respective genetic purity with regard to DAS-81910-7 cotton and their respective water content. The certified value is traceable to the International System of Units (SI).

5) The uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 %, estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

Aad-12	Aryloxyalkanoate dioxygenase-12
ANOVA	Analysis of variance
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EC	European Commission
ERM®	Code for certified reference materials from the Joint Research Centre
EU	European Union
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
g	Relative centrifugal force
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurement
EDTA	Ethylenediaminetetraacetic acid
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
JRC	Joint Research Centre
k	Coverage factor
LOD	Limit of detection
M	Molar, defined as number of moles per liter
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
n.c.	Not calculated
Pat	Phosphinothricin acetyltransferase
PCR	Polymerase chain reaction
PSA	Particle size analysis
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference Material
rpm	Revolutions per minute
RT	Room temperature
s	Sample standard deviation
$s_{\bar{x}}$	Standard deviation of the estimate of the mean (also referred to as standard error of the estimate of the mean)
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
SI	International System of Units
s_{rel}	Relative standard deviation (also referred to as RSD)
s_{wb}	Within-unit standard deviation; an additional index "rel" is added as appropriate
t	Time
t_i	Time point for each replicate
t_{sl}	Chosen shelf life
t_{tt}	Chosen transport time
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation in quantitative PCR
TE	Tris-EDTA
u	Standard uncertainty
U	Expanded uncertainty

u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by the intermediate precision of the method; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
ν	Degrees of freedom
V-KFT	Volumetric Karl Fischer Titration
v/v %	Volume / volume percentage
$\overline{x}$	Arithmetic mean
$\overline{y}$	Mean of all results of the homogeneity study

1 Introduction

1.1 Background: need for the CRM

The European Union has legislation which regulates the placing on the market of any food or feed which consists of, contains, or is produced from genetically modified organisms (GMOs). These items are referred to as genetically modified food and feed and require authorisation for marketing in the European Union. They are also required to be labelled if they contain more than 0.9 % of GMOs [5]. This labelling threshold is applicable for the adventitious presence of GMOs, whilst GMOs that are intentionally added need to be labelled independently from any threshold. However, feed may contain 0.1 (m/m) % of a GMO for which an authorisation process is pending, or for which authorisation in the EU has expired [6]. These thresholds require the development and validation of reliable methods for GMO quantification, and the production of reference materials for calibration or quality control of these methods.

Dow AgroSciences LLC (Indianapolis, US) developed the genetically modified (GM) DAS-81910-7 cotton event (unique identifier code DAS-8191Ø-7, following Commission Regulation (EC) No 65/2004 [7]) as a transgenic herbicide tolerant crop. The DAS-81910-7 cotton event was developed by *Agrobacterium tumefaciens* mediated transformation and has been modified to express aryloxyalkanoate dioxygenase-12 (Aad-12) and phosphinothricin N-acetyltransferase (Pat), which confer tolerance to the herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and glufosinate respectively [8]. In 2016 Dow AgroSciences LLC (Indianapolis, US) commissioned the European Commission's Joint Research Centre, Directorate F – Health, Consumers and Reference Materials (Geel, BE) to produce a certified reference material (CRM) for the quantification of DAS-81910-7 cotton. The CRM produced by the JRC received the code ERM-BF440 and is composed of five CRMs containing different mass fractions of DAS-81910-7 cotton. Like previous CRM productions, the codes used for the different concentrations of the mass fraction of DAS-81910-7 cotton followed the labelling pattern where the ERM-BF440a and ERM-BF440b are the pure non-GM and GM materials, and ERM-BF440c, d and e are 0.1 %, 1 % and 10 % materials, respectively.

1.2 Choice of the material

The set of CRMs ERM-BF440 was produced from milled GM and non-GM seeds. Seeds (in contrast to the grains) were selected as the source of raw material because of their high degree of purity.

1.3 Design of the CRM project

The genetic purity with respect to the DAS-81910-7 cotton event of the non-GM and GM cotton seeds has been investigated.

Alongside the pure non-GM material ERM-BF440a and the pure GM material ERM-BF440b, mixtures of non-GM and GM cotton powder were prepared gravimetrically. The first mixed material ERM-BF440e was prepared by mixing pure GM with non-GM cotton powder. ERM-BF440d was prepared by further dilution of ERM-BF440e, and ERM-BF440c was prepared by further dilution of ERM-BF440d, in both cases with non-GM cotton powder.

The different mass fractions of ERM-BF440 were certified using a gravimetric approach, the details of which are described in Section 6.

2 Participants

2.1 Provider of raw material and quantification method

Dow AgroSciences LLC (Indianapolis, US) provided the raw materials.

Dow AgroSciences Ltd. (Abingdon, UK) provided the event-specific quantitative PCR method under a confidential agreement with JRC.

2.2 Project management, processing, analytical measurements and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials

(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM, and to ISO/IEC 17025 for GMO quantification, BELAC No. 268-TEST)

3 Material processing and process control

3.1 Origin of the starting material

Dow AgroSciences LLC (Indianapolis, US) supplied the Joint Research Centre, Directorate F – Health, Consumers and Reference Materials (JRC, Geel, BE) with non-GM cotton seeds and DAS-81910-7 cotton seeds to prepare candidate CRMs. According to the information provided by Dow AgroSciences Ltd (Abingdon, UK), the DAS-81910-7 cotton seeds are homozygous. After arrival, the seeds were stored at (4 ± 3) °C in the dark until processing.

The genetic purity with respect to the DAS-81910-7 cotton event of the GM cotton seeds was assessed at the JRC by analysing 216 randomly selected seeds for the presence of the DAS-81910-7 cotton GM event. Genomic DNA was extracted from 216 plants grown from individual seeds using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). An event-specific quantitative PCR method to detect the DAS-81910-7 cotton event received from Dow AgroSciences was first validated in-house and afterwards applied by the JRC to verify the presence of the DAS-81910-7 event in the plants grown from the seeds. Once a method has been internationally validated by the European Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), it will be published on the EURL homepage [9]. Genomic DNA extracted from pure DAS-81910-7 cotton powder was used as positive control using the CTAB method (Annex A). Amplification and detection was performed on a QuantStudio 7 quantitative PCR system following the protocol for JumpStart™ Taq ReadyMix™ for Quantitative PCR (Sigma-Aldrich, BVBA/SPRL Overijse, B) [10]. The results showed that all the GM seeds tested gave a signal for the presence of the DAS-81910-7 cotton event. Statistical analysis of the 216 measurements (Poisson distribution for rare events) revealed that the GM cotton seed batch had a genetic purity > 98.6 % (95 % level of confidence). The genetic purity was set at 99.3 % the half width between 100 and 98.6 %. The statistical genetic purity >98.6 (95 % level of confidence) was taken into consideration during the estimation of the uncertainties associated with the certified values of the CRMs (Section 6.2).

The genetic purity of the non-GM seed batch with respect to the DAS-81910-7 cotton event was investigated using the processed seed powder. Five bottles of ERM-BF440a were randomly selected and the DNA was extracted from two samples (extraction replicates) taken from each bottle ($N = 5$, $n = 2$). Each DNA extract was then analysed in 3 replicates by quantitative PCR method, with a limit of detection (LOD) of 0.07 g/kg. This analysis did not detect the DAS-81910-7 cotton event (Section 3.4). The LOD of the event-specific

quantitative PCR method was taken into consideration when the certified value of ERM-BF440a was calculated (Section 7).

3.2 Processing and process control

All cotton seeds received by the JRC (Geel, BE) were rinsed with water, drained, and dried on trays in the drying chamber of a freeze-dryer at 25 °C for 20 h (Epsilon 2-100D, Martin Christ, Osterode, DE).

Approximately 30 kg of non-GM cotton seeds and 20 kg of DAS-81910-7 cotton event seeds were used for the production of the ERM-BF440.

The GM and non-GM base materials were processed separately into powders. Cross-contamination between them and contamination with foreign DNA were avoided by treating all the contact surfaces with DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) before exposure to the materials and using clean laboratory clothing. An in-house validation study had previously proven that the solution degraded DNA effectively under the given conditions.

The cotton seeds were frozen overnight in liquid nitrogen in approximately 4 kg portions in stainless steel containers and were subsequently milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). The mill was maintained below -90 °C throughout the process. The feeding speed of the mill was optimised to ensure that the seeds were milled to the required particle size. The powder was then cold sieved with a 710 µm stainless steel mesh on a sieving machine equipped with an ultra-sound sieving aid (Russel Finex, London, UK). After sieving, the powder was maintained at (-20 ± 3) °C. The remaining powder from each base material was cold mixed in a DynaMIX CM200 (WAB, Muttenz, CH) for 1 h to homogenize the distribution of the different types of cotton seed tissues, since it is known that the milling and sieving processes result in separation of the various seed tissues from each other.

The residual water mass fractions of the non-GM and GM powders were measured by volumetric Karl Fischer titration (V-KFT, 841 KFD Titrando, Metrohm, Herisau, CH), as (57.6 ± 8.2) g/kg and (53.0 ± 7.5) g/kg respectively (N = 1, n = 3), with the expanded uncertainty calculated using a coverage factor k = 2. To facilitate gravimetric mixing, the water content of the powders was further reduced by drying them overnight under vacuum in the freeze-dryer at 25 °C. The final water mass fractions of the non-GM powder and the GM powder were measured as (7.8 ± 1.1) g/kg and (9.8 ± 1.4) g/kg, respectively (N = 1, n = 3), with the expanded uncertainty calculated using a coverage factor k = 2, (Table 1).

Also, the particle size distribution for both powders was measured based on their deconvoluted laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and were then compared (Figure 1). The cumulative volume distribution of the particles derived from laser diffraction data is based on their equivalent spherical diameter, i.e. the diameter of the particles derived from the volume occupied upon their rotation. The mean particle diameter of the non-GM and GM powder materials was 183.0 µm ± 25.0 µm (s) and 165.5 µm ± 17.1 µm (s), respectively. However, since most particles are not perfectly spherical, the calculated volume of the particles based on their diameter will overestimate the mean particle size. Therefore, a three-point specification of the particle size distribution (N = 1, n = 5) was calculated, consisting of the equivalent sphere diameters where 10 %, 50 % and 90 % of the total volume distribution have a smaller particle size (Table 1). These size classes are denoted as X_{10}, X_{50} and X_{90}, respectively. A t-test showed with 95 % confidence that there was no significant difference between the X_{10}, X_{50}, X_{90} values and between the mean particle diameter of the non-GM and GM cotton powders. It was concluded that the non-GM and GM powder materials were sufficiently similar with respect to their particle size distribution and they could be processed further without introducing a bias which could subsequently affect the extractability of the DNA.

Table 1: The water mass fraction determined by V-KFT and additionally the particle diameter and particle size distribution based on the deconvoluted laser diffraction patterns of the powder materials

Powder material	Water mass fraction [g/kg]		Mean particle diameter [µm]		Particle size distribution X_{10} [µm]		Particle size distribution X_{50} [µm]		Particle size distribution X_{90} [µm]	
	$\bar{x}$	U	$\bar{x}$	s	$\bar{x}$	U	$\bar{x}$	U	$\bar{x}$	U
Non-GM powder	7.8 [1)]	1.1	183.0 [2)]	25.0	30.5 [3)]	6.3	149.7 [3)]	24.9	382.3 [3)]	78.0
GM powder	9.8 [1)]	1.4	165.5 [2)]	17.1	28.4 [3)]	5.9	143.0 [3)]	23.8	337.1 [3)]	68.8

[1)] Mean of one sample (N = 1, n = 3). The associated expanded uncertainty (U) with a coverage factor k = 2 has been estimated during validation of the V-KFT method on cotton powder.

[2)] Mean of one sample (N = 1, n = 5) with the sample standard deviation.

[3)] Mean of one sample (N = 1, n = 5). Given are the equivalent sphere diameters for which 10 %, 50 % or 90 % of the particles in the volume distribution have a smaller particle size. The associated expanded uncertainty (U) with a coverage factor of k = 2 has been estimated during validation of the particle size measurement method.

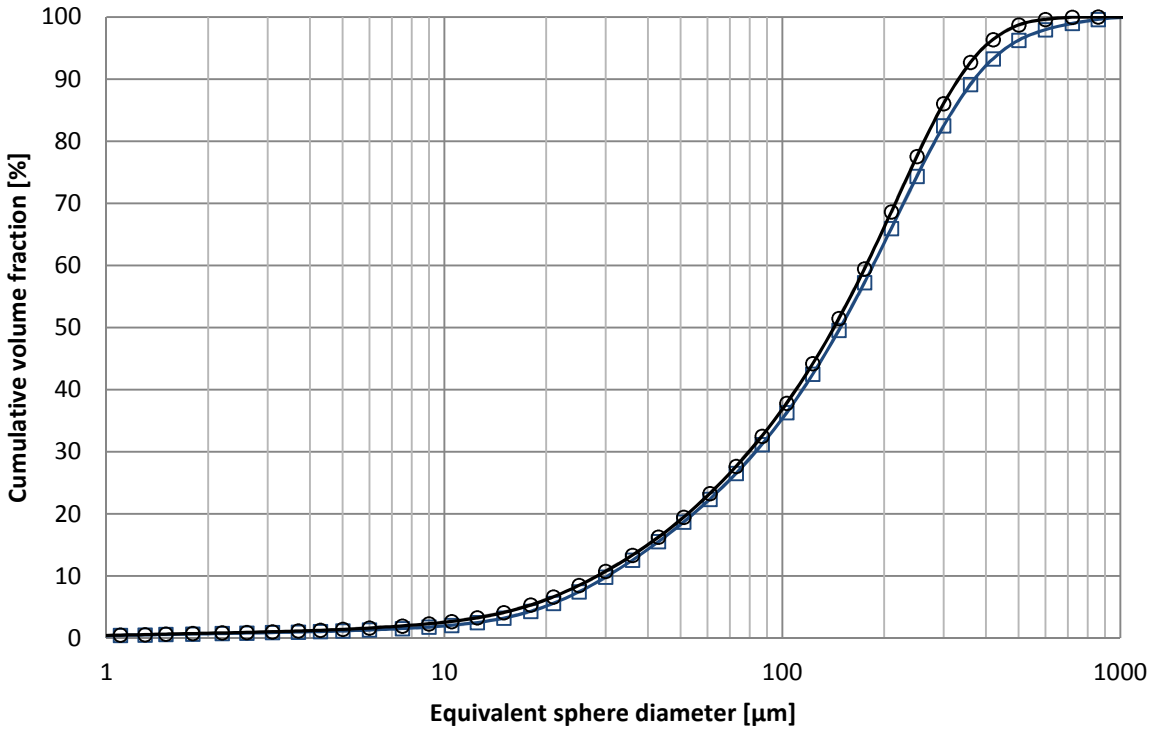

Figure 1: Volume-based cumulative distribution of equivalent sphere diameters in the GM powder (○) and non-GM powder (□) analysed by laser diffraction (N = 1, n = 5). The total particle volume for each material is set as 100 %.

The milled base materials were used to prepare the blank material for DAS-81910-7 cotton (non-GM cotton seed powder), the pure GM DAS-81910-7 cotton material and three mixtures at nominal mass fraction levels of 1, 10 and 100 g/kg DAS-81910-7 cotton event. The term "nominal" is used for the target value during the processing whereas the value assigned after completion of the certification process is called certified value.

All the materials were treated according to the same procedure and strict measures were taken to avoid cross-contamination. The powder materials were weighed using a calibrated balance (MSU-8202-S, Sartorius, Göttingen, DE) with an intermediate precision, determined during calibration and expressed as standard uncertainty (u), of 0.02 g. Calibration of the balance is performed on an annual basis by an external company (accredited under ISO/IEC 17025). The performance of the balance was verified before use on a daily basis by using in-house reference weights. The masses of the non-GM and GM powders, which are theoretically needed to reach a certain nominal mass fraction, were calculated corrected for their respective water content. Portions of the powder materials were weighed into a container and mixed for 1 h by using a Dyna-MIX CM 200 (WAB, Muttenz, CH). The material with a nominal DAS-81910-7 cotton mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM powder materials. Similarly, the material with a nominal DAS-81910-7 cotton mass fraction of 10 g/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder and the material with a nominal mass fraction of 1 g/kg was produced by further dilution of the 10 g/kg GM powder with pure non-GM powder. At each mixing step, the water mass fraction of the materials was taken into account (Table 6). During the certification process, the gravimetric preparation was the basis for the calculation of the certified DAS-81910-7 cotton mass fraction for the three powder mixtures (Section 6).

An automatic filling device (All-Fill Sandy, UK) was used to fill the powders into 10 mL amber glass bottles. To avoid cross contamination the equipment was cleaned between two mass fraction levels and the first 30 bottles of each batch were discarded as an additional precaution. The blank material was filled first, followed by the mixtures with increasing mass fraction with the pure GM material filled last. Lyophilisation inserts were automatically placed in the bottle necks. The bottles were then placed in a freeze-dryer (Epsilon 2-100D, Martin Christ, Osterode, DE) to provide an argon atmosphere, and were closed inside the freeze-dryer with the help of a hydraulic device. Capping and labelling took place in a capping and labelling assembly from Bausch & Ströbel and BBK, respectively (Ilshofen and Beerfelden, both DE). Colour-coded caps were used to facilitate the identification of the different mass fraction levels of DAS-81910-7 cotton event: nominal 0 g/kg = silver (BF440a), nominal 1000 g/kg = black (BF440b), nominal 1 g/kg = gold (BF440c), nominal 10 g/kg = red (BF440d), nominal 100 g/kg = brown (BF440e), consistent with the cap colours of previous JRC CRMs for GMOs. Each of the bottles was identified by a numbered label indicating the ERM code and the unit number according to filling order. After the inventory and the selection of bottles for future analysis according to a random stratified sampling scheme, the remaining bottles were stored in the dark at 4 ± 3 °C.

Ten randomly selected bottles from each of the powder materials were measured by V-KFT to determine the residual mass fraction of water in the candidate CRMs. The results are summarised in Table 2.

Table 2: Water mass fractions of candidate ERM-BF440 CRMs determined by V-KFT (N = 10, n = 1). The associated expanded uncertainty (U) has been estimated during validation of the V-KFT method on cotton powder

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	U (k = 2)
ERM-BF440a	10.7	1.4
ERM-BF440b	9.9	1.3
ERM-BF440c	8.6	1.1
ERM-BF440d	7.6	1.0
ERM-BF440e	8.9	1.2

The particle size distribution in the candidate CRMs was determined based on the deconvoluted laser diffraction pattern of the constituent powders. Five randomly selected bottles from each of the candidate CRMs were analysed twice (N = 5, n = 2) and 98.86 % of the particles had a size below 1020 µm (Figure 2). The mean particle diameters and standard deviations of the mean, measured by laser diffraction, were 194.3 µm ($s_{\bar{x}}$ = 24.5 µm), 160.8 µm ($s_{\bar{x}}$ = 8.2 µm), 191.6 µm ($s_{\bar{x}}$ = 19.6 µm), 191.4 µm ($s_{\bar{x}}$ = 31.4 µm) and 178.4 µm ($s_{\bar{x}}$ = 5.9 µm) for ERM-BF440a, b, c, d and e, respectively.

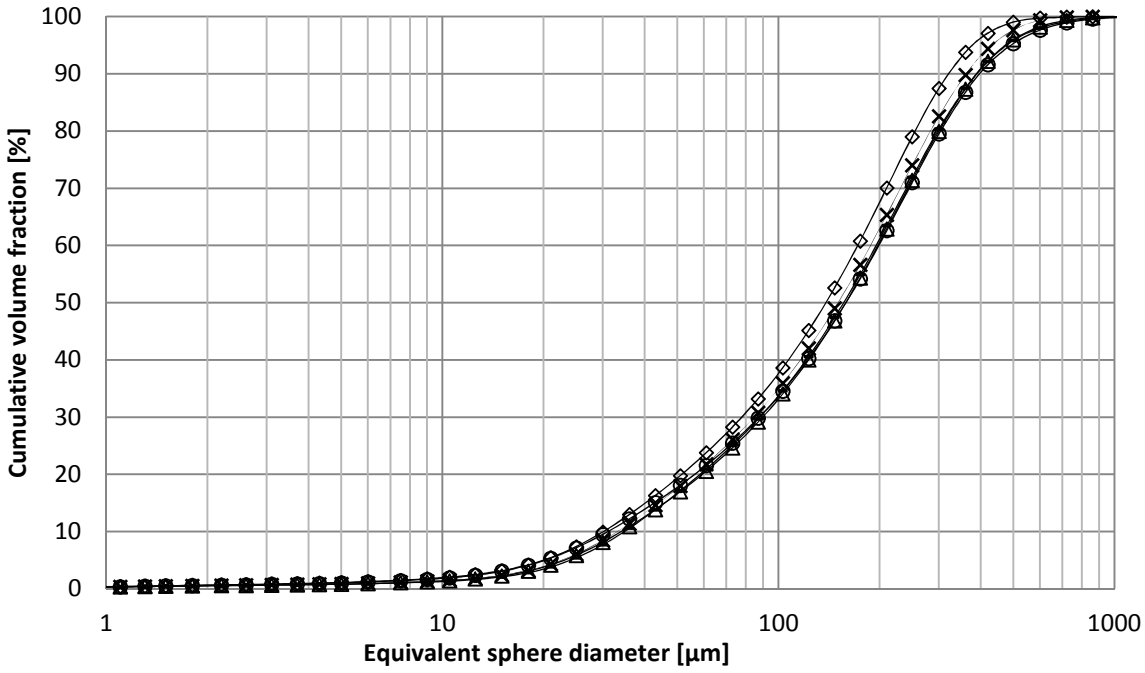

Figure 2: Volume based cumulative distribution of particle size in ERM-BF440a (○), ERM-BF440b (◇), ERM-BF440c (△), ERM-BF440d (-) and ERM-BF440e (x) analysed by laser diffraction (N = 5, n = 2). The total particle volume for each preparation is set as 100 %.

3.3 Total DNA content of the powder materials

Three of the described CRMs are mixtures of GM and non-GM cotton seed powders, produced gravimetrically and intended to be used for quality control or calibration of quantitative measurements of the genomic DNA, following DNA extraction and purification. Any DNA mass fraction difference in the non-GM and GM base materials will lead to a shift of the measurement results obtained with e.g. quantitative PCR. However in the case of cotton, the classical fractionation method developed by Ogur and Rosen [11] does not allow the quantification of the DNA mass fraction. The extraction with perchloric acid leads to the formation of a red colour which interferes with the spectrophotometer measurement of the 2-deoxyriboses [11, 12]. Therefore, no proof could be delivered that the certified GM powder mass fractions are equal to the corresponding transgenic and target taxon-specific DNA copy number ratio.

It has to be understood that the ERM-BF440 has been developed to set a common reference point for the implementation of EU legislation on GMO thresholds and labelling. DNA extractability may depend on the DNA extraction method selected. Furthermore, the assigned certified GM mass values of the prepared mixtures can only be reproduced by quantitative PCR, if the possible difference in DNA extractability of GM and non-GM cotton powders is taken into account. The difference in the extractability can be for example, attributed to the difference in the size of the non-GM and GM seeds and the difference in particle size of the non-GM and GM powders. During the visual inspection the seeds were found equal in size and the non-GM and GM powder materials were sufficiently similar with respect to their particle size distribution (Section 3.2).

Gel electrophoresis was used to check the integrity of the DNA. DNA was extracted from 200 mg samples taken from each of the candidate CRM, ERM-BF440a, ERM-BF440b, ERM-BF440c, ERM-BF440d and ERM-BF440e, using a CTAB tip-20 DNA extraction method (Annex A). None of the samples showed DNA degradation (data not shown).

3.4 Consistency measurements

As a control for the gravimetric preparations, the mass fraction of DAS-81910-7 cotton event in the mixed materials ERM-BF440c, ERM-BF440d and ERM-BF440e was measured using the event-specific in-house validated quantitative PCR method provided by Dow AgroSciences.

At the JRC genomic DNA was extracted by a validated CTAB extraction method (Annex A) using 200 mg powder samples. After the extraction, the DNA was diluted in a TE-low buffer solution (pH 8.0, 1 mmol/L Tris and 0.01 mmol/L EDTA) and used to produce calibration curves for the cotton-specific gene and the transgene. The quantitative PCR test was calibrated with genomic DNA extracted from pure DAS-81910-7 cotton powder. For the calibration curve of the cotton-specific gene, the DNA was used undiluted (approximately 200 ng DNA per 25 µL reaction) and diluted up to 200-fold. For the calibration curve of the transgene, the DNA was used in concentration of approximately 50 ng DNA per 25 µL reaction and was then subsequently diluted up to 2500-fold. The efficiency of the amplification was assessed from the slope of the regression line between the calibrants' mass fractions of DAS-81910-7 cotton event and from the Cq-values. The LOD of the PCR method was calculated as 3.3-fold *s* of the lowest calibration point at which s_{rel} was below 25 %. The results of the quantification of DAS-81910-7 cotton event are shown in Table 3. The quantitative PCR measurements confirmed that the mass fractions of the DAS-81910-7 in the mixed materials ERM-BF440c, d and e were consistent with the gravimetrical approach used for their preparation. No independent calibration was carried out and therefore the data in Table 3 can only be used for confirmation of the consistency of the powder dilutions during processing.

Table 3: Quantification of the DAS-81910-7 cotton mass fraction in the candidate CRMs by event-specific quantitative PCR using genomic DNA from pure DAS-81910-7 cotton seed powder for calibration

Candidate CRM	DAS-81910-7 cotton mass fraction [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF440a	< 0.07 [1) 2)]	-
ERM-BF440b	992 [1)]	20 [5)]
ERM-BF440c	1.2 [3)]	0.1 [6)]
ERM-BF440d	11.3 [4)]	0.5 [6)]
ERM-BF440e	109 [1)]	4 [6)]

[1)] Mean of 2 samples (extraction replicates) from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 quantitative PCR replicates.

[2)] The value was below the LOD determined during method validation (0.07 g/kg).

[3)] Mean of 3 samples (extraction replicates) from each of 12 randomly selected bottles (N = 12, n = 3), with each sample measured in 3 quantitative PCR replicates.

[4)] Mean of 2 samples (extraction replicates) from each of 12 randomly selected bottles (N = 12, n = 2), with each sample measured in 3 quantitative PCR replicates.

[5)] Uncertainty of the measurement includes the repeatability.

[6)] Uncertainty of the measurement includes the repeatability and intermediate precision.

4 Homogeneity

A key requirement for any CRM aliquotted into units is the equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty associated with the certified value, although it is not necessarily relevant whether the variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34:2009 [1] requires RM producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

This homogeneity study was planned together with the measurements to control the gravimetric preparations and the short-term stability of the CRMs (Sections 3.4 and 5.1). These data were appropriate for investigating homogeneity since they had been obtained under intermediate precision conditions on bottles taken randomly from the entire batch and analysed in a randomised order. Two extraction replicates per bottle were analysed for ERM-BF440d and ERM-BF440e, compared to three for ERM-BF440c. The number of extraction replicates was chosen based on the intermediate precision of the in-house validated method, such that the standard uncertainty for the within-unit variation would be less than 25 %. Homogeneity of the blank powder was demonstrated in the course of the test for the genetic purity of the raw materials by taking two extraction replicates from 5 randomly selected bottles of ERM-BF440a. The homogeneity of ERM-BF440b is related to the purity study of the seeds. As all 216 tested seeds gave a signal for the DAS-81910-7 cotton event, using the statistical analysis (Poisson distribution for rare events) with 95 % level of confidence the batch was considered to be homogeneous (Section 3.1).

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRMs are valid for all bottles containing the material, within the stated uncertainties.

For the between-unit homogeneity test, the number of bottles selected corresponds to approximately the cube root of the total number of bottles produced. Therefore, 12 bottles were selected for ERM-BF440c and 14 bottles for ERM-BF440d. To facilitate both the homogeneity studies and the short-term stability study, 15 bottles were selected for ERM-BF440e. For each candidate CRM, a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12, 14 and 15 groups respectively (with a similar number of bottles) and one bottle was randomly selected from each group. For ERM-BF440c, three independent samples (extraction replicates) were taken from each bottle whilst for the candidate CRMs with higher GM mass fractions, ERM-BF440d and ERM-BF440e, two independent samples (extraction replicates) were taken from each bottle. All samples were analysed by quantitative PCR. Due to the number of PCR plates required, the measurements were performed under intermediate precision conditions. Samples were analysed in a randomised manner to be able to separate a potential analytical trend from a trend in the filling sequence. The results are shown in the figures in Annex B.

Regression analyses were performed to evaluate potential trends in the filling sequence. No trends were observed at a 95 % confidence level.

In addition, regression analyses were performed to evaluate potential trends in the analytical sequence. There were no significant trends (95 % confidence level) in the analytical sequence detected.

The datasets for ERM-BF440c, ERM-BF440d and ERM-BF440e were assessed for consistency using Grubbs outlier tests at a 99 % confidence level on the individual results and on the unit means. No outlying individual results neither unit means were detected using the double Grubbs outlier test.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method intermediate precision if the individual samples were representative of the whole unit.

Evaluation by ANOVA requires mean values per unit which follow at least a unimodal distribution and results for each unit that have approximately the same standard deviation. Too few data are available for the unit means to make a clear statement about the distribution. Therefore, it was visually checked whether all individual data followed a unimodal distribution using histograms and normal probability plots.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean squares between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method intermediate precision, was calculated as described by Linsinger *et al.* [13]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method intermediate precision ($s_{wb,rel}$), between-unit standard deviation ($s_{bb,rel}$) and maximum hidden inhomogeneity ($u^*_{bb,rel}$) were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 1}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{\nu_{MSwithin}}}}{\bar{y}} \qquad \textbf{Equation 3}$$

MS_{within}	within-unit mean square from an ANOVA
$MS_{between}$	between-unit mean square from an ANOVA
$\bar{y}$	mean of all results of the homogeneity study
n	mean number of replicates per unit
$\nu_{MSwithin}$	degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 4.

Table 4: Results of the homogeneity study

Candidate CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
ERM-BF440c	10.3	n.c.[1)]	3.2	3.2
ERM-BF440d	8.6	n.c.[1)]	3.7	3.7
ERM-BF440e	7.4	n.c.[1)]	3.2	3.2

[1)] n.c: cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation (s_{bb}) can be used as an estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity (u_{bb}).

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used for analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 200 mg sample intake. This sample intake gives acceptable intermediate precision, demonstrating that the within-unit inhomogeneity no longer contributes to the analytical variation at this sample intake.

ERM-BF440a and ERM-BF440b are pure non-GM and GM materials, respectively. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. However, based on the quantitative PCR measurements carried out on these two powders it was concluded that also for these two pure materials the suitable minimum sample intake for quantitative PCR is 200 mg.

5 Stability

Time, temperature and light were regarded as the most relevant influences on the stability of the materials. The influence of light was minimised by storing the materials in amber glass bottles which reduce light exposure. In addition, materials were stored in the dark and dispatched in boxes, thus removing any possibility of degradation due to light. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for the dispatch of the materials to the customers (short-term stability). During transport, especially in the summer, temperatures of up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The ERM-BF440e material was selected for the short-term stability study because it is a mixture of both GM and non-GM base material and allows assessing the stability of each base material. Moreover, it is the mixture with the highest GM mass fraction, enabling the best method intermediate precision ($s_{wb,rel}$) of all three mixtures (Table 4). The short-term stability study was carried out using an isochronous design [14]. In this approach, samples of ERM-BF440e were stored for a defined length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under intermediate precision conditions.

ERM-BF440 is a dried cotton seed powder, which has been prepared in a similar manner to previous GMO CRM cotton powders produced by the JRC and which have similar water content and particle size distribution. Therefore, the data obtained from the stability monitoring of previous cotton GMO CRMs were used to assess the long-term stability of ERM-BF440, and to estimate the uncertainty associated with storage of this CRM.

5.1 Short-term stability study

For the short-term stability study, units of ERM-BF440e were stored at 4 °C, 18 °C and 60 °C for each of 1, 2 and 4 weeks, whereupon they were moved to the reference temperature (-70 °C). Units representing the time point of 0 weeks were kept at a reference temperature (-70 °C). Five units per storage time and temperature were selected using a random stratified sampling scheme. From each unit, two extraction replicates were measured by quantitative PCR. The measurements were performed under intermediate precision conditions with respect to the PCR plates, and a randomised sequence was used to differentiate any potential analytical trend from a trend over storage time.

The data were evaluated individually for each of the three temperatures tested. The results were screened for outliers using the single and double Grubbs test at a 99 % confidence level. No statistical outliers were detected in any of the studies for any of the temperatures.

Also, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated to test for potential increases/decreases of the DAS-81910-7 cotton mass fraction due to the simulated shipping conditions. The slopes of the regression lines were tested for statistical significance. There were no trends that were statistically significant on a 95 % confidence level for any of the temperatures.

The material can thus be dispatched without further precautions under ambient conditions.

The results of the measurements are shown in Annex C.

5.2 Long-term stability study

Data from the stability monitoring program for GMO CRMs were available. Previously released cotton powder CRMs were analysed for their GM mass fraction with 36 data points over a period of 11 years. On each occasion, measurements were performed simultaneously on one PCR plate, using DNA extracted from units stored at the normal storage temperature (4 °C) and at a reference temperature (-70 °C). Each of these studies can be viewed as a two-point isochronous study. The evaluation was based on the GM mass fraction ratio of results of the samples stored at 4 °C and -70 °C.

The outcome of the short-term stability studies of ERM-BF440e showed equivalent short-term stability at 4° C, 18 °C and 60 °C. Taking into account the demonstrated short term stability at 60 °C, a condition which is more likely to show any instability of the material, the short-term stability studies confirm that the data obtained from the stability monitoring of other cotton GMO CRMs produced and stored in the same way as ERM-BF440, can be used to estimate the stability uncertainty contribution for ERM-BF440 relating to the storage of the CRM (Section 5.1).

The long-term stability data for cotton GMOs were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No statistical outliers were detected, and the results were retained for the estimation of u_{lts}.

The data were also evaluated against storage time and regression lines were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected at a 95 % confidence level.

The material can, therefore, be stored at 4 °C.

The results of the measurements are shown in Annex D.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out the degradation of materials, even in the absence of statistically significant trends. It is, therefore, necessary to quantify the potential degradation that could be hidden by the method intermediate precision, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation.

The uncertainties of stability during dispatch and storage were estimated, as described in [15]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 4}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 5}$$

s_{rel} relative standard deviation of all results of the stability study

t_i time elapsed at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at 60 ºC)

t_{sl} chosen shelf life (24 months at 4 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for 1 week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the stability monitoring program for cotton GMO CRMs. The uncertainty contribution describes the possible degradation during 24 months storage at 4 °C.

The results of these evaluations are summarised in Table 5.

Table 5: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of 4 °C and 24 months

Candidate CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-BF440	0.6	0.8

After the certification study, the materials will be included in the JRC's regular stability monitoring programme to assess their further stability.

6 Characterisation

For the purpose of RM certification, material characterisation is the term used to describe the process of determining the certified value of a reference material.

The five candidate CRMs, under the label ERM-BF440, are cotton powder materials processed from non-GM and GM seeds. While ERM-BF440a was prepared from the pure non-GM material and ERM-BF440b from the pure GM material, the other candidate CRMs of the ERM-BF440 series are gravimetrically diluted mixtures of the pure non-GM and GM cotton seed powders. ERM-BF440 is certified for the mass fraction of the DAS-81910-7 cotton event. Gravimetric mixing was the method of choice based on a primary method of measurement confirmed by PCR analysis.

6.1 Genetic purity of the materials

The genetic purity with respect to the DAS-81910-7 cotton event of the GM and non-GM batches used for the processing of the candidate CRMs was investigated to calculate the certified value.

No indication was found that the GM cotton material contained seeds that were negative for the event DAS-81910-7 cotton (Section 3.1).

The powder used for the production of ERM-BF440a did not contain traces of DAS-81910-7 cotton above the LOD of the quantitative PCR method used (Sections 3.1 and 3.4). The certified value for ERM-BF440a is therefore based on the LOD of the quantitative PCR method, as determined during in-house method validation.

The eventual adventitious presence of other GM events in both the GM and non-GM cotton powders was verified by using a qualitative PCR-based ready-to-use multi-target analytical system for GM detection developed by JRC (Ispra, IT) [16]. This test was performed at the JRC by using a pre-spotted 96-well plate containing primers and probes for simultaneous detection of targeting 44 individual specific GM events (Maize: E3272, E98140, BT11, BT176, DAS 59122, GA21, MIR162, MIR604, MON 810, MON 863, MON 87460, MON 88017, MON 89034, NK603, T25, TC1507; Soya: DAS 40278, A2704, A5547, CV127,

DP 305423, DP 356043, FG72, GTS 40-3-2, MON 87701, MON 89788; Rapeseed: T45, GT73, MS1, MS8, RF1, RF2, RF3, Topas 19/2; Cotton: E281, E3006, GHB119, GHB614, LL Cotton25, MON 1445, MON 15985, MON 531, MON 88913, T304) and the primers and probes for the specific detection of 4 taxon-specific assays for maize (*hmg*), soya bean (*Lec*), rapeseed (*CruA*) and the cotton reference gene (*Sah7*). Any stacked events derived from the single-insert GMOs included in the system would also be detected.
The results indicated that both cotton powders used for the production of ERM-BF440 did not contain any of the above tested GM events and were only positive for the taxon-specific detection for cotton (*Sah7*).
Since no evidence of contamination was found in the non-GM and the GM materials, 100 % genetic purity was used for the calculation of the certified mass fraction of DAS-81910-7 cotton in the powder mixtures. The difference between the statistically established genetic purity of at least 98.6 % (Section 3.1) and the 100 % genetic purity was taken into account in the uncertainty calculation.

6.2 Mass fractions and their uncertainties

The certified mass fraction values are based on the mass fractions of mixed GM and non-GM powder, corrected for their water mass fractions and taking into account the powder's genetic purity with regards to the DAS-81910-7 cotton event. The values were calculated according to the following equations:

$$\text{GM mass fraction [g/kg]} = \frac{m_{\text{GM,dry}}}{m_{\text{GM,dry}} + m_{\text{nonGM,dry}}} \times 1000 \qquad \textbf{Equation 6}$$

$$m_{\text{GM,dry}} = m_{\text{GM}} \times (1 - \text{WMF}_{\text{GM}}) \qquad \textbf{Equation 7}$$

$$m_{\text{nonGM,dry}} = m_{\text{nonGM}} \times (1 - \text{WMF}_{\text{nonGM}}) \qquad \textbf{Equation 8}$$

$m_{\text{GM,dry}}$	mass [g] of the GM powder corrected for its water mass fraction
$m_{\text{nonGM,dry}}$	mass [g] of the non-GM powder corrected for its water mass fraction
m_{GM}	mass [g] of the GM powder used for the dilution
m_{nonGM}	mass [g] of the non-GM powder used for the dilution
WMF_{GM}	water mass fraction of the GM powder [g/g]
$\text{WMF}_{\text{nonGM}}$	water mass fraction of the non-GM powder [g/g]

The data supporting the calculation of the mass fractions of DAS-81910-7 cotton are summarised in Table 6.

Table 6: Subsequent mixing of pure DAS-81910-7 GM cotton seed powder with pure non-GM powder to prepare the ERM-BF440c, d and e materials

Candidate CRM	GM powder [1]			Non-GM powder [1]		Mixtures
	GM Mass fraction [g/kg]	Water mass fraction ± U (k = 2) [g/kg]	Mass [g]	Water mass fraction ± U (k = 2) [g/kg]	Mass [g]	Calculated GM mass fraction [g/kg]
ERM-BF440e	1000.0 [2]	9.8 ± 1.4	400.74	7.8 ± 1.1	3599.29	100
ERM-BF440d	100.0 [3]	9.4 ± 1.3	400.53	7.8 ± 1.1	3599.47	10.0
ERM-BF440c	10.0 [4]	9.3 ± 1.3	400.50	7.8 ± 1.1	3599.50	1.00

[1] Calculations of the mass fraction of DAS-81910-7 cotton in the powder mixtures are based on a 100 % genetic purity with regard to DAS-81910-7 cotton of the non-GM and GM powder materials.

[2] Pure DAS-81910-7 GM cotton seed powder was used for the preparation of ERM-BF440e.

[3] GM powder mixture ERM-BF440e was used for the preparation of ERM-BF440d.

[4] GM powder mixture ERM-BF440d was used for the preparation of ERM-BF440c.

The uncertainties of the certified DAS-81910-7 cotton mass fractions (u_{char}) have several components, i.e. the uncertainty arising from weighing ($u_{char,1}$), the uncertainty of the determination of the water mass fraction ($u_{char,2}$), and the uncertainties associated with the determination of the genetic purity concerning the DAS-81910-7 cotton event of the non-GM and GM powder materials ($u_{char,3}$ and $u_{char,4}$, respectively). Based on a statistical analysis of the probability distribution of finding a negative seed in the GM raw material, it was concluded that the genetic purity of the event DAS-81910-7 cotton event in this CRM, was higher than 98.6 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value (Table 7).

Table 7: Uncertainty budgets for the mass fractions of DAS-81910-7 cotton in ERM-BF440

Candidate CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]				Combined standard uncertainty u_{char} [g/kg]
		$u_{char,1}$ [1)]	$u_{char,2}$ [2)]	$u_{char,3}$ [3)]	$u_{char,4}$ [4)]	
ERM-BF440a	0	n.a.[5)]	n.a.[5)]	0.0202	n.a.[5)]	0.0202
ERM-BF440b	1000	n.a.[5)]	n.a.[5)]	n.a.[5)]	4.0034	4.0034
ERM-BF440c	1	0.0004	0.0014	0.0202	0.0040	0.0206
ERM-BF440d	10	0.0029	0.0121	0.0202	0.0400	0.0465
ERM-BF440e	100	0.0206	0.0984	0.0202	0.4003	0.4133

[1)] Standard uncertainty of the mass determination, based primarily on the uncertainty of the balance and the number of weighing steps required.

[2)] Standard uncertainty of the water mass fraction determination by V-KFT.

[3)] Standard uncertainty of the genetic purity estimation of the non-GM powder material (LOD = 0.07 g/kg), based on the half-width of the interval between 0 and 0.07 g/kg, divided by the square root of 3 (rectangular distribution).

[4)] Standard uncertainty of the genetic purity estimation of the GM raw material (> 98.6 %), based on the interval between 98.6 % and 100 % divided by the square root of 3 (rectangular distribution).

[5)] not applicable

6.3 Consistency measurements

Quantitative PCR measurements confirmed that no mixing errors were made during the preparation of the candidate CRMs (Section 3.4). Additionally, gel electrophoresis proved that the DNA was not degraded during the processing of the candidate CRMs (Section 3.3).

7 Value assignment

Certified values are values that fulfil the highest standards of accuracy assessment. Therefore full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

The assigned certified values are based on the masses of dried powder of GM seeds and non-GM seeds used in the gravimetrical preparation. The masses of the powders were corrected for their respective water mass fractions during the preparation of the materials (Table 6).

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} (Section 6.2), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5.3). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,rel}$) with a coverage factor k given as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{char,rel}^2 + u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2} \qquad \textbf{Equation 9}$$

- u_{char} was estimated as described in Section 6.2.
- u_{bb} was estimated as described in Section 4.1.
- u_{sts} and u_{lts} were estimated as described in Section 5.3.

For the blank material, the LOD of the method was used to describe the 95 % confidence interval of the certified mass fraction of the event (< 0.07 g/kg). This was supported by the high genetic purity with regards to the DAS-81910-7 cotton event of the non-GM material and the absence of a mixing step; calculating the U_{CRM} for the blank material on the basis of the only quantifiable standard uncertainty ($u_{char,3}$) gives a value of U = 0.04 g/kg (assuming k = 2), which is below the certified < 0.07 g/kg value. The LOD is, therefore, a conservative estimate of the certified value and its uncertainty.

For the pure GM material, the statistically calculated genetic purity of the GM seed batch (Section 3.1) was used to describe the 95 % confidence interval of the certified mass fraction of the event (> 986 g/kg). Calculating U_{CRM} for the pure GM material on the basis of the only quantifiable standard uncertainty ($u_{char,4}$) gives a value of U = 8 g/kg (assuming k = 2), which is less than the difference between the nominal value (1000 g/kg) and the certified value (> 986 g/kg). The statistically calculated genetic purity is, therefore, a conservative estimate of the certified value and its uncertainty.

For the three mixtures, the certified values were established by gravimetry, and the measured mass fraction values had an expanded uncertainty with a coverage factor of 2, established during calibration of the balance. Therefore, the same coverage factor (k = 2) was used to obtain the expanded uncertainties for ERM-BF440c, d and e.

The certified values and their uncertainties are summarised in Table 8.

Table 8: Certified values and their uncertainties for ERM-BF440

CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{lts} [g/kg]	U_{CRM} [3] [g/kg]
ERM-BF440a	< 0.07 [1]	0.0202	n.a.[4]	n.a.[4]	n.a.[4]	-
ERM-BF440b	> 986 [2]	4.0034	n.a.[4]	n.a.[4]	n.a.[4]	-
ERM-BF440c	1.00	0.0206	0.0320	0.0060	0.0080	0.08
ERM-BF440d	10.0	0.0465	0.3700	0.0600	0.0800	0.8
ERM-BF440e	100	0.4133	3.2001	0.6000	0.8000	7

[1] With 95 % confidence, the certified value is below this level.

[2] With 95 % confidence, the certified value is above this level.

[3] Expanded (k = 2) and rounded uncertainty

[4] not applicable

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The identity of the measurand is based on the documentary traceability to the DAS-81910-7 cotton event, (Biosafety Clearing House, record ID 108872) [8].

Quantity value

The traceability chain for the certified values for the pure non-GM and GM CRMs, ERM-BF440a and ERM-BF440b, respectively, are based on the genetic purity assessment using a validated event-specific DAS-81910-7 cotton quantitative PCR method and verified equipment.

The traceability chain for the certified values for the mixtures in ERM-BF440c, d and e is based on the use of calibrated balances and a thorough control of the weighing procedure.

The certified values are therefore traceable to the International System of Units (SI).

8.2 Commutability

ERM-BF440 were prepared gravimetrically from non-GM and GM seed powders with the aim to implement the corresponding EU legislation for food and feed which uses threshold in mass fractions.

ERM-BF440 is intended to be used as calibrant or quality control for quantitative PCR measurements of the cotton GM event DAS-81910-7 in food and feed. Consequently, this certified reference material is establishing, together with the measurement method validated by the EURL-GMFF [9], the arbitrary reference system for quantification of DAS-81910-7 cotton. Therefore, commutability, which is a crucial characteristic for reference materials in case that a different measurement method would be applied, does not have to be considered here.

9 Instructions for use

9.1 Safety and protection of the environment

The usual laboratory safety measures apply. The material is for *in-vitro* use only. As it is a milled material, it does not contain any viable seeds.

9.2 Storage conditions

The materials should be stored at 4 ± 3 °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material is hygroscopic. The user should close CRM bottles immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened bottles.

9.3 Minimum sample intake

The minimum sample intake for a DNA extraction is 200 mg cotton powder.

ERM-BF440a and ERM-BF440b are pure non-GM and GM materials. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. Nevertheless it is recommended that the same sample intake is used as for the mixed materials to obtain a significant amount of DNA.

9.4 Use of the certified value

The intended use of these materials is for calibration or quality control of methods for the identification and quantification of genetically modified DAS-81910-7 cotton in food and feed. As with any reference material, they can be used for establishing control charts and validation studies.

The user is reminded that this reference material is certified for its DAS-81910-7 cotton mass fraction and should be used for measurements expressed in mass fractions. The exact relationship between the certified GM powder mass fractions and the corresponding DNA copy number ratio is not known. Changing the measurement unit from mass fraction to copy number per haploid genome equivalent, for instance, requires the use of a conversion factor that is only an approximate value, thereby adding additional uncertainty to the measurement result.

Use as a calibrant

If this matrix material is used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty. Furthermore, it should be noted that using the same material for calibration and quality control limits the effectiveness of the control, as calibrant and quality control material are based on the same raw materials. If this is unavoidable, it is recommended that different mass fraction levels of ERM-BF440 are used for calibration and for quality control.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, https://crm.jrc.ec.europa.eu/graphics/cms_docs/erm1_english.pdf [17]).

When assessing the method performance, the measured values of the CRMs are compared to the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{\mathrm{meas}}^2 + u_{\mathrm{CRM}}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty (u_{Δ}), using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %.
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

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Annexes

Annex A: CTAB-tip20 DNA extraction method (as modified in-house)

Solutions and reagents

1. CTAB buffer A
   - 20 g/L CTAB
   - 1.4 M NaCl
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA, pH 8.0
2. CTAB buffer B
   - 10 g/L CTAB
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA, pH 8.0
3. Chloroform-Octanol (24:1 v/v)
4. 1.2 M NaCl
5. Proteinase K, 20 mg/mL
6. RNase A, 100 mg/mL
7. Qiagen buffer G2
8. Qiagen Genomic-tip 20/G columns
9. Qiagen QBT equilibration buffer
10. Qiagen QC washing buffer
11. Qiagen QF elution buffer
12. Isopropanol
13. 70 % (v/v) Ethanol
14. TE low buffer, 1 mmol/L Tris and 0.01 mmol/L EDTA, pH 8.0

DNA extraction protocol

a) Weigh 200 mg cotton powder into a 2 mL microcentrifuge tube
b) Add 1.3 mL of CTAB Buffer A + 5 µL RNase A + 6.5 µL Proteinase K and mix by vortexing
c) Incubate 45 min at 60 °C, shaking at 1400 rpm
d) Centrifuge for 10 min at 16000 x *g* at room temperature (RT)
e) Transfer 750 µL of supernatant to a 2 mL microcentrifuge tube containing 1 mL of chloroform:octanol (24:1 v/v)

f) Mix thoroughly by inverting, incubate 5 min at RT
g) Centrifuge for 10 min at 16000 x *g* at RT
h) Transfer 600 µL of supernatant to a new 2 mL microcentrifuge tube containing 1400 µL of CTAB Buffer B
i) Mix thoroughly by inverting, incubate 20 min at RT
j) Centrifuge for 15 min at 16000 x *g* at RT
k) Discard the supernatant by pipetting and conserve the pellet
l) Add 200 µL of 1.2 M NaCl
m) Incubate 5 min at 50 °C, shaking at 1400 rpm
n) Add 1.6 mL of G2 buffer + 2.5 µL of RNase A + 20 µL of Proteinase K
o) Incubate 1 h at 50 °C, shaking at 650 rpm
p) Centrifuge 5 min at 16000 x *g* at RT
q) Equilibrate a Qiagen Genomic-tip 20/G column with 1 mL of QBT buffer
r) Apply the sample to the equilibrated Genomic-tip 20/G column by pipetting
s) Wash the genomic-tip 20/G column with 3 mL of QC buffer
t) Elute the genomic DNA with 1 mL of QF buffer (pre-warmed at 50 °C) and collect the DNA in a 2 mL tube
u) Add 700 µL of isopropanol to each tube, invert 10 times
v) Centrifuge for 20 min at 10000 x *g* at 4 °C, discard the supernatant by pipetting
w) Wash the pellet with 1 mL of 70 % (v/v) ethanol
x) Centrifuge 10 min at 13000 x *g* at 4 °C
y) Discard the supernatant by pipetting and air-dry the pellet for 10 min
z) Dissolve the DNA pellet in 80 µL of TE Low buffer preheated at 50 ºC and incubate 10 min at 50 °C while shaking at 500 rpm. Let the pellet to dissolve completely overnight at 4 °C and store the samples at + 4 °C (short-term) or at -20 °C (long-term).

Annex B: Results of the homogeneity measurements

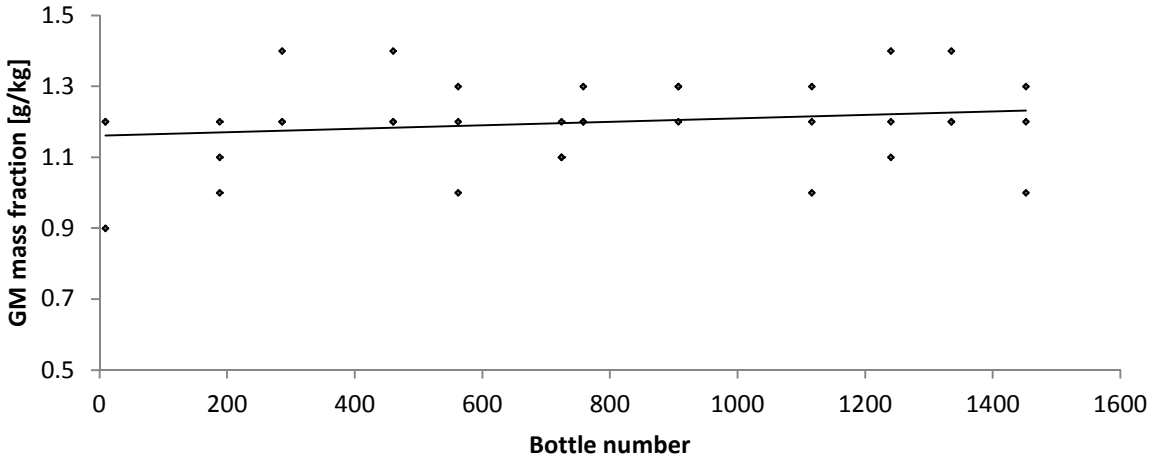

Figure B1: Quantitative PCR measurement results for ERM-BF440c. Three samples (extraction replicates) were measured from each of 12 randomly selected bottles (*N* = 12, *n* = 3), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

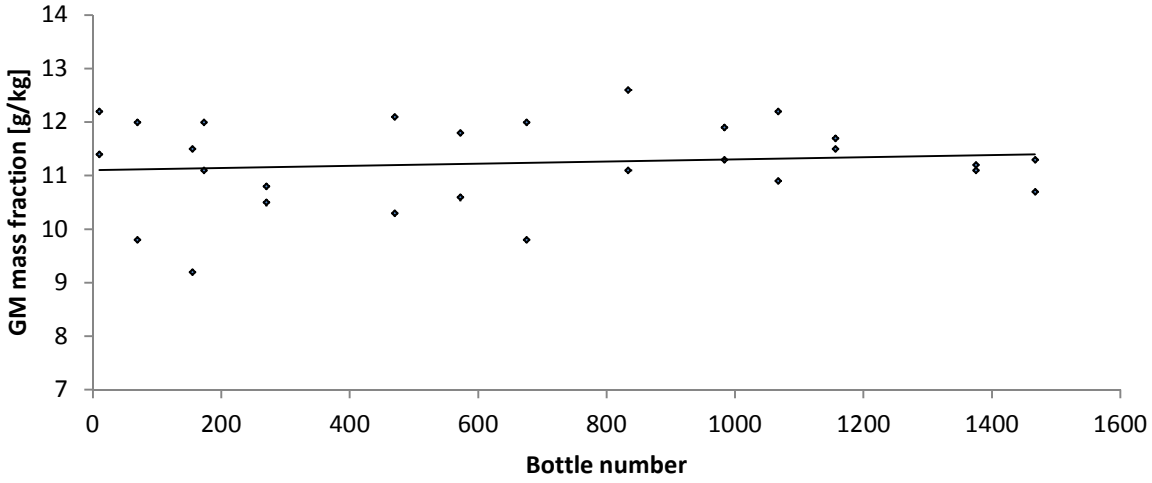

Figure B2: Quantitative PCR measurement results for ERM-BF440d. Two samples (extraction replicates) were measured from each of 14 randomly selected bottles (*N* = 14, *n* = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

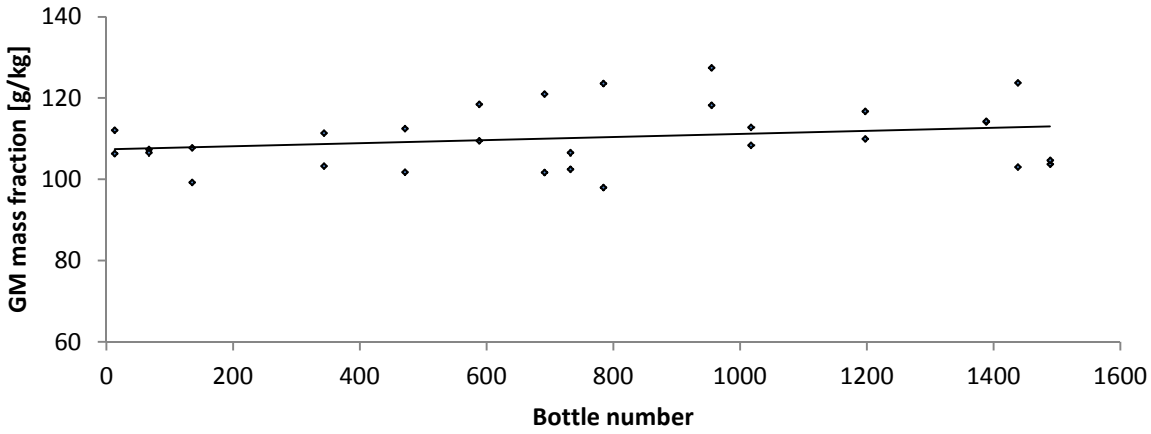

Figure B3: Quantitative PCR measurement results for ERM-BF440e. Two samples (extraction replicates) were measured from each of 15 randomly selected bottles (N = 15, n = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

Annex C: Results of the short-term stability measurements

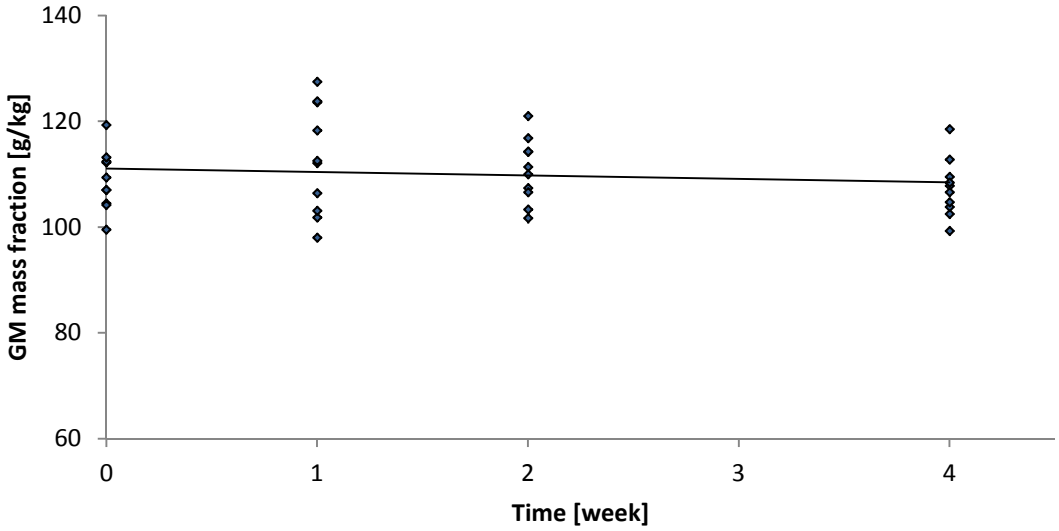

Figure C1: Quantitative PCR measurement results for ERM-BF440e during short-term stability testing at 4 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (*N* = 5, *n* = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

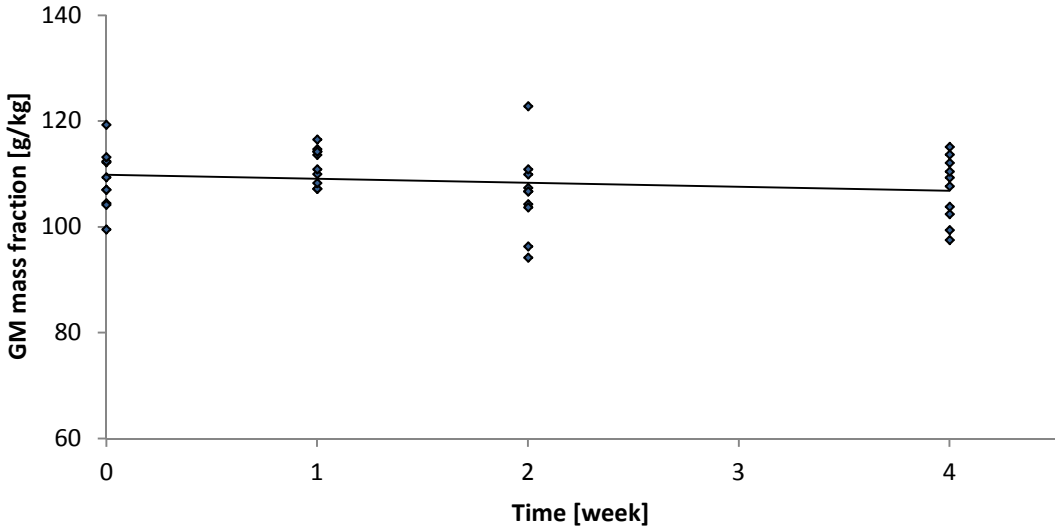

Figure C2: Quantitative PCR measurement results for ERM-BF440e during short-term stability testing at 18 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (*N* = 5, *n* = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

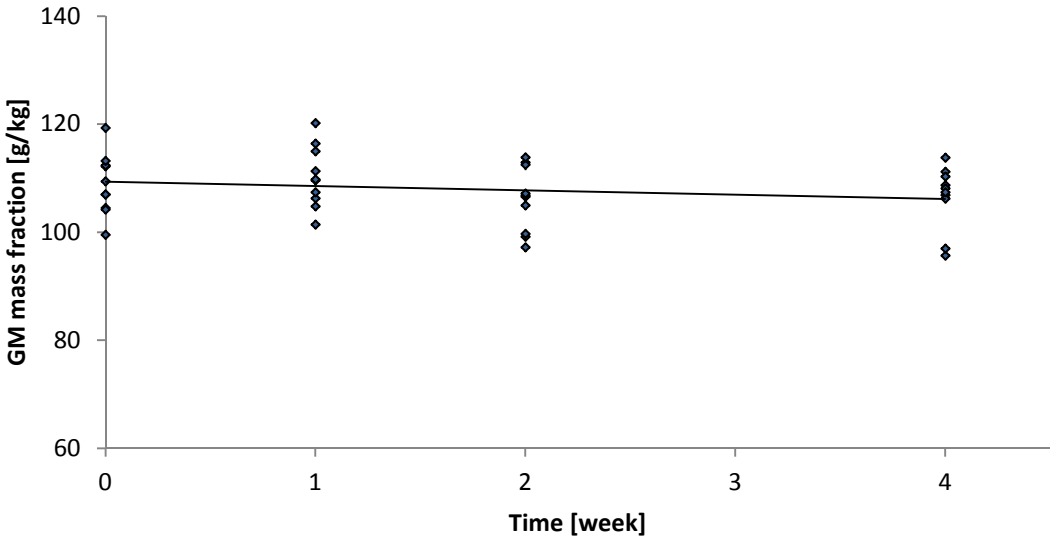

Figure C3: Quantitative PCR measurement results for ERM-BF440e during short-term stability testing at 60 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

Annex D: Results of the long-term stability measurements

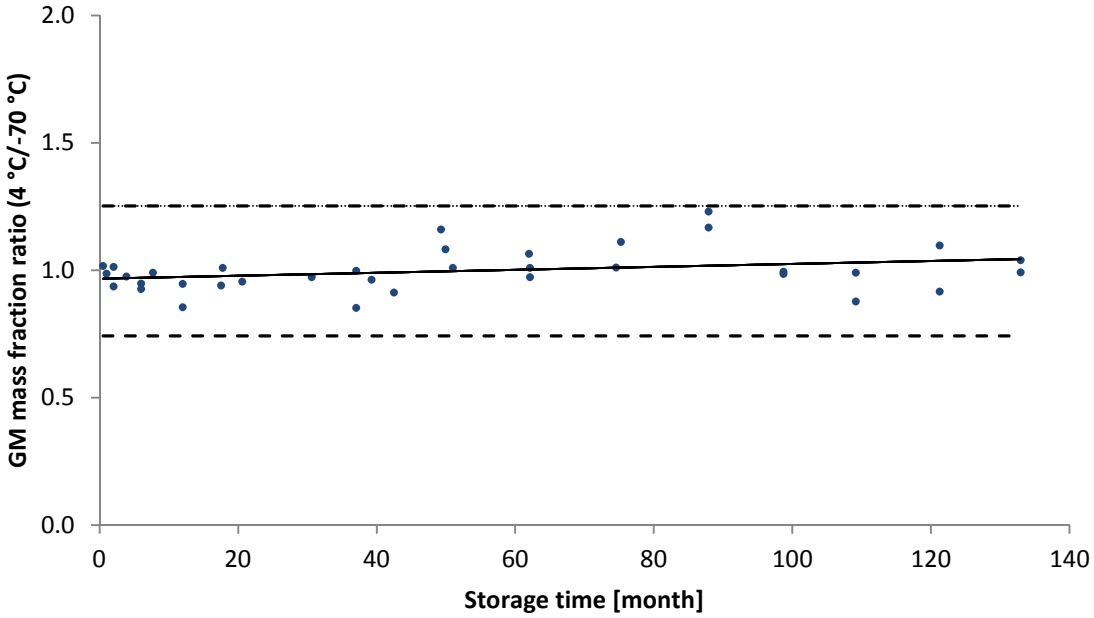

Figure D1: Quantitative PCR measurement results of ERM-BF440e (1, 2 and 4 weeks) and ERM-BF422, ERM-BF428, and ERM-BF429 (data from the post-certification monitoring). The dashed lines give the limits of 3*s* obtained for the measurement results. The straight line is a least-squares linear regression for all data points.

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