Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of Reference Materials of Cotton Seed Powder with Different Mass Fractions of the Cotton Event GHB119

Certified Reference Materials ERM®-BF428 (ERM®-BF428a, ERM®-BF428b, ERM®-BF428c)

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Certification of Reference Materials of Cotton Seed Powder with Different Mass Fractions of the Cotton Event GHB119

Certified Reference Materials ERM®-BF428 (ERM®-BF428a, ERM®-BF428b, ERM®-BF428c)

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this report 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.

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GLOSSARY

ANOVA	analysis of variance
CRL-GMFF	Community Reference Laboratory for Genetically Modified Food and Feed
CRM	Certified Reference Material
DNA	deoxyribonucleic acid
ERM®	trademark of European Reference Materials
GHB119	GM cotton (*Gossypium hirsutum)* event GHB119
GM	genetically modified
GMO	genetically modified organism
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
KFT	Karl Fischer titration
LOD	limit of detection
N	number of samples analysed
n	number of subsamples analysed
n.a.	not applicable
PCR	polymerase chain reaction
PSA	particle size analysis by laser diffraction
s	standard deviation
s_{bb}	standard deviation between bottles
SI	International System of Units
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation during real-time PCR
U	expanded uncertainty
u_{bb}	standard uncertainty related to the between-bottle heterogeneity
u^*_{bb}	standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
u_{char}	standard uncertainty related to the characterisation
u_{lts}	standard uncertainty related to the long-term stability of the material
$\overline{x}$	calculated average value

1 Introduction and design of the project

Legislation in the European Union demands the labelling of food and feed products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs) [1]. This is the labelling threshold level for GMOs that are authorised in accordance with Community legislation. In general, this demands on the one hand the development and validation of reliable methods for GMO quantification, and on the other hand the production of reference materials for the quality control and calibration of these methods.

GMO Certified Reference Materials (CRMs) from the Institute for Reference Materials and Measurements (IRMM, Geel, BE) have been produced from genetically modified (GM) powder and non-GM powder, both produced from seed material. Beside a non-GM pure material, gravimetric mixtures of non-GM and GM cotton powder were prepared by dry-mixing, a first material by mixing non-GM and GM cotton powder and a second one by further dilution of the mixture with non-GM cotton powder. This certification report describes the certification of the matrix CRMs for their mass fraction of GHB119 cotton. A certification of the DNA copy number ratio is envisaged for the future, allowing the implementation of European Commission Recommendation (EC) No 787/2004 to express the content of GM food and feed as the percentage of transgenic DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes [2].

Bayer BioScience NV (Gent, BE) has developed the GM cotton event GHB119. Following Commission Regulation (EC) No 65/2004 [3], the GHB119 event received the unique identifier code BCS-GHØØ5-8.

In 2008, the IRMM was asked by Bayer BioScience to develop and produce a series of reference materials for the quantification of GHB119 cotton. The resulting CRM has been named ERM-BF428 and is composed of a set of three CRMs with different mass fractions of the genetically modified GHB119 cotton seed.

2 Participants

- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium (BELAC, 268-TEST)*

* Measurements within the scope of accreditation to ISO/IEC 17025.

3 Base material and processing

3.1 Characterisation of the base materials

For the preparation of the CRMs, Bayer BioScience supplied conventional cotton seeds and GHB119 cotton seeds to IRMM. After arrival, the cotton seeds were stored (4 ± 3) °C in the dark until use. Twenty-five kg of non-GM cotton seeds and 10 kg of GHB119 cotton seeds were used for the processing of ERM-BF428.

The purity of the GM cotton seed batch was verified at IRMM by analysing 200 randomly selected GM seeds for the presence of the GM event GHB119. In order to avoid influences from adhering dust particles on the analytical results, seedlings were grown and genomic DNA was extracted from the leaves using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). Quantitative real-time PCR was performed according to the event-specific real-time PCR method delivered under confidentiality agreement to IRMM. This method will be published after completion of its international validation on the homepage of the Community Reference Laboratory for GM food and feed [4]. Genomic DNA extracted from pure GHB119 cotton seed powder was used for calibration. Detection was done on an ABI7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [5]. The results, summarised in Table 1, showed that all plants from the GM seed batch gave a signal for presence of the GHB119 event. Statistical analysis (Poisson distribution for rare events) revealed that the GM cotton seed batch had a genetic purity of > 98.5 % (95 % confidence level), respectively.

Table 1: Genetic purity of the GM cotton seed batch used for the processing of ERM-BF428 with respect to GM event GHB119, the resulting lot purity is given at a 95 % confidence level and expressed as number fraction

Batch	Number of seeds tested	Number of GHB119 positives	Number of GHB119 negatives	Lot purity [%]
GM	200	200	0	> 98.5 %

The calculated lot purity of the GM seed batch was taken into account for the estimation of the uncertainties on the certified values of the reference materials (Section 7.2).

The purity of the non-GM seed batch was investigated after processing of the powder. Real-time PCR measurements on the non-GM cotton seed powder were performed, with a limit of detection (LOD) of 0.2 g/kg the method did not detect the event GHB119 (Section 3.5). Additionally 53 DNA extracts from individual seedlings of the non-GM seed batch were analysed. Also here, no evidence for the presence of the event GHB119 was found.

3.2 Processing of the base materials

The GM and non-GM base materials were processed separately. Cross-contamination and contamination with foreign DNA were avoided using glove box systems and clean laboratory clothing. All contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to exposure to the base materials. An in-house validation study had proven beforehand that the solution degraded DNA effectively under the given conditions. If required, the base powders were stored for short time periods in closed plastic containers.

The cotton seeds received by IRMM were cleaned from cotton fibres by an acid delinting treatment. Neutralisation of the acid was afterwards done by addition of NaOH. A thorough two step washing procedure was carried out at IRMM to remove the residues from the black seeds. The cotton seeds were rinsed for about 15 minutes with water, drained, and dried over night under vacuum in a freeze drier (Epsilon 2-65D, Osterode, DE) at 30 °C. The rinsing, draining and drying step was repeated and resulted in visually clean seeds. The cotton seeds received had a water mass fraction of 60 g/kg with an expanded uncertainty of 9 (k = 2), after the two drying steps the seeds had a remaining water mass fraction of 29-34 g/kg with an expanded uncertainty of 5 (k = 2). The dried seeds were then milled using a high impact mill with a triangular ribbed open grinding track in order to obtain the ground base material. The high impact mill was flushed with nitrogen gas throughout the milling process and milling was interrupted if the temperature rose above 47 °C. An additional drying at 30 °C was carried out under vacuum in a freeze drier to further reduce the water content of the once ground base material. This resulted in a water mass fraction of between 7 and 8 g/kg with an expanded uncertainty of 2 (k = 2). The powders were ground a second time applying a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). Prior to the second grinding step the cotton seed powder was frozen overnight in approximately 4 kg portions in metal containers in liquid nitrogen. The vibrating mill was also cooled down to process the powders at a temperature below -95 °C. The feeding speed of the Palla mill was set in such a way as to ensure an efficient grinding with respect to the particle size obtained. After milling, the powder was kept at room temperature to reach a thermal equilibrium. It was then sieved with a 1000 µm stainless steel sieve on a Russel Finex (London, UK). A mass of about 1 %, representing mainly a part of the seed covers from the cotton seed which did not get milled, was removed by sieving from the non-GM and GM batch. Each ground base material was mixed in a DynaMIX CM200 (WAB, Basel, CH) for 30 min to improve equal distribution of the different parts of the cotton tissues separated by the milling process. The final water mass fraction of the non-GM powder was 19.1 ± 2.7 g/kg (U, k = 2) and 17.4 ± 2.5 g/kg for the GM powder (Table 5). The powders were stored in closed plastic containers until further use.

3.3 Gravimetric preparation of mixtures

The ground base materials were used to produce a blank material for GHB119 and two mixtures containing different mass fractions of GHB119 cotton seed powder in non-GM cotton seed powder at nominal levels of 10 and 100 g/kg. All three materials, including the blank powder, were treated according to the same procedure. The powder materials were weighed using a calibrated balance with an intermediate precision, expressed as relative standard uncertainty, of 0.1 %. The starting materials were combined in one container, mixed in the DynaMIX CM200 mixer for 30 min, and further homogenised in a propeller mixer for an additional 2 min. The blank material was processed first, followed by the mixtures. For the preparation of the mixtures the masses of the non-GM and GM powder were corrected for their respective water mass fractions. The nominal mass fraction of 100 g GHB119/kg was produced by mixing pure GM with pure non-GM ground base materials. The mass fraction containing nominal 10 g GHB119/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder; at each mixing step, the water mass fraction of the mixed materials was taken into account (Table 5). The gravimetric

preparation formed the basis for the calculation of the mass fraction of the powders (Section 6).

3.4 Bottling

The powders were bottled in 10 mL brown glass vials using an automatic filling device. The first 30 bottles of each batch were discarded as an additional precaution against carry-over contamination. Lyophilisation inserts were automatically placed in the bottle neck. Before final closure of the vials, air was evacuated in a freeze-drier and replaced by argon. The vials were closed inside the freeze-drier with the help of a hydraulic device and then sealed with aluminium caps to prevent accidental opening during storage and transport. Colour-coded caps were used for easy identification of the different levels of GHB119: nominal 0 g/kg = silver (BF428a), nominal 10 g/kg = red (BF428b), nominal 100 g/kg = brown (BF428c), consistent with the cap colours of previous IRMM CRMs. Each of the vials was identified by a numbered label indicating the ERM code (Figure 1). Following the inventorying and the selection of vials for future analysis according to a random stratified sampling scheme, the bottles were brought to a storage room for long-term storage in the dark at (4 ± 3) °C.

Figure 1: Prototype labels for the ERM-BF428 series. The denotation 'blank' was used for the nominal 0 g/kg GHB119 cotton powder (BF428a), while BF428b and BF428c refer to the nominal 10 g/kg and 100 g/kg GHB119 cotton, respectively.

3.5 Processing control

The residual mass fraction of water in ten randomly selected bottles from each of the powder materials was determined by volumetric Karl Fischer titration (KFT). The results are summarised in Table 2. As a result of the hygroscopic nature of the powders, it is recommended to close the vials immediately after taking a sample.

Table 2: Water mass fraction in candidate CRMs ERM-BF428 determined by volumetric KFT (*N* = 10, *n* = 1)

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	*U (k = 2)*
ERM-BF428a	17.8	2.4
ERM-BF428b	19.0	2.5
ERM-BF428c	16.9	2.3

Five randomly selected bottles from each of the powder materials were analysed for their particle volume distribution based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). It is important to understand that the cumulative volume distribution of particles derived from laser light scattering data, is based on their equivalent spherical diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. Since most particles are presumably not perfectly spherical, the volume-based presentation of the PSA data is, therefore, overestimating the average particle size. From each bottle, 3 subsamples were analysed. The powders had a maximum particle volume below 735 µm and very similar distribution profiles (Figure 2). The average particle volume and *s* (*N* = 5, *n* = 3), calculated by the PSA software, was 144 ± 10 µm, 149 ± 5 µm and 142 ± 8 µm for ERM-BF428a, b and c, respectively.

It was concluded from the results of particle volume analysis that the powders are sufficiently fine for an adequate extraction of genomic DNA [6].

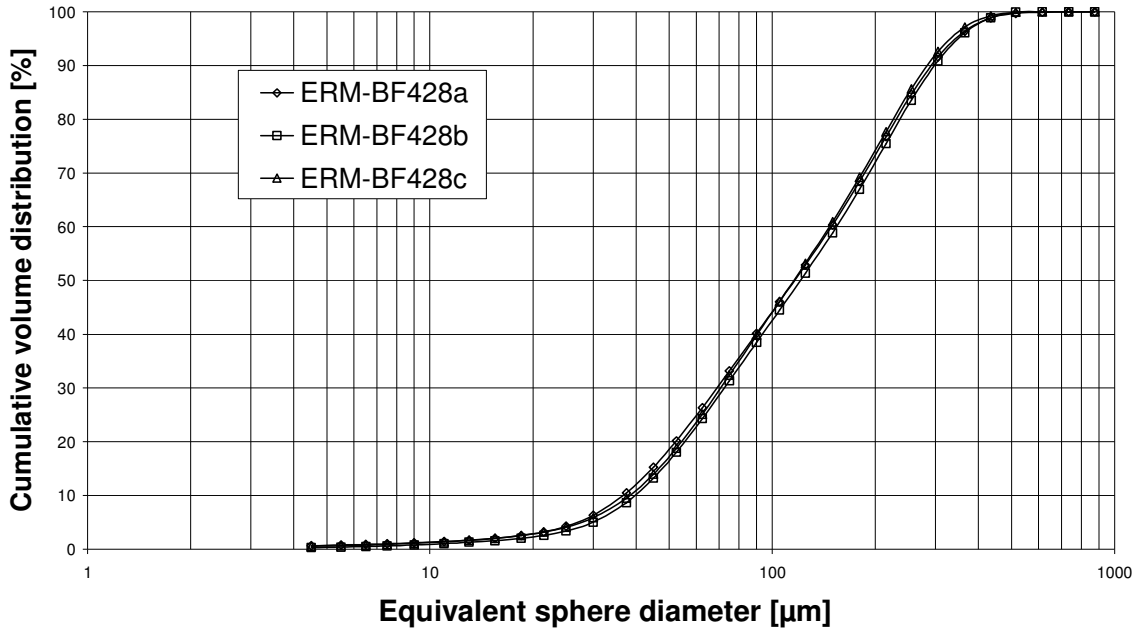

Figure 2: Average particle volume distribution in ERM-BF428 analysed by PSA (*N* = 5, *n* = 3).

Two of the described CRMs are mixtures of GM and non-GM cotton seed powders, produced gravimetrically and used for quality control or calibration of quantitative measurements on the level of the genomic DNA, following DNA extraction. It therefore is essential that the DNA mass fractions in both pure base materials do not deviate significantly from each other. The classical fractionation method developed by Ogur and Rosen [7] did not lead to reliable results. DNA spiking experiments had indicated varying recoveries. The extraction with perchloric acid leads to the formation of a red colour which interferes with the spectrophotometer measurement of the 2-deoxyriboses [7, 8]. 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.

The DNA integrity was controlled by gel electrophoresis. From 3 subsamples of each of the processed powder materials for ERM-BF428a, BF428b and BF428c, DNA was extracted from 500 mg powder using a CTAB/Genomic DNA Tip 20 DNA extraction method validated by the CRL GMFF for cotton seed [9]. Approximately 0.4 µg DNA was made visible on an agarose gel with a mass concentration of a 7.5 g/L. A 1 kb DNA ladder (New England Biolabs, Inc., Ipswich, MA, USA) was used to track the DNA size. None of the bands showed DNA degradation (Figure 3).

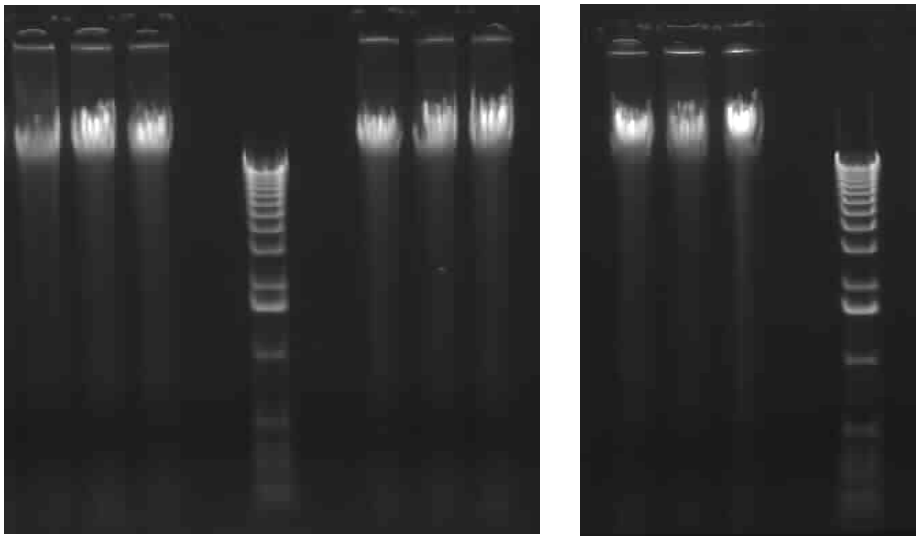

Figure 3: DNA integrity of ERM-BF428a, b and c powder materials was made visible on an agarose gel after DNA extraction with CTAB/Genomic DNA Tip 20 (*N* = 1, *n* = 3) using a 1 kb DNA ladder.

As a control for the gravimetric preparations, the mass fraction of GHB119 cotton in all three CRMs was verified by the confidential real-time PCR method targeting the transgenic DNA insertion in this cotton. Genomic DNA was extracted from 500 mg powder samples using a CTAB/Genomic DNA Tip 20 DNA extraction method validated by the CRL GMFF [9]. The real-time PCR test was calibrated with genomic DNA extracted from the pure GHB119 cotton powder, and afterwards diluted with water to produce a calibration curve ranging from 4-times diluted to 2000-times diluted and from undiluted to 100-times diluted for the transgenic gene and target taxon-specific gene, respectively. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of GHB119 and the obtained Ct-values; for all standard curves, the efficiency was within the limits of the real-time PCR control chart. The limit of detection (LOD) was calculated using the calibration curve approach [10]. The results of the

quantification of GHB119 for the three candidate CRMs are shown in Table 3. Quantification of the mass fraction of GHB119 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF428. However, as no independent calibration was carried out the data displayed in Table 3 can be used for confirmation of the processing, but do not necessarily resemble the true value.

Table 3: Quantification of the GHB119 cotton mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure GHB119 seed powder for calibration

Candidate CRM	GHB119 cotton mass fraction [g/kg]	U (k = 2) [g/kg]
ERM-BF428a	< 0.2 [1)]	-
ERM-BF428b	9.6 [2)]	2.9 [3)]
ERM-BF428c	102.6 [2)]	8.4

[1)] Average for 2 subsamples from each of 10 random bottles (N = 10, n = 2), with each subsample measured in three replicates. The obtained value is below the LOD determined during method validation (0.2 g/kg).
[2)] Average for 2 subsamples from each of 15 random bottles (N = 15, n = 2), with each subsample measured in three replicates.
[3)] Data obtained were found to be non-normally distributed; as a consequence the uncertainty estimation given here is not based on the in-house method validation but on the approach explained in [14] and in Section 4.1.

4 Homogeneity

In order to ensure that the CRMs are sufficiently homogeneous, two strategies were followed: validation of the mixing procedure and homogeneity control of the produced two mixtures. The adequacy of the dry-mixing technology for the preparation of cotton seed powder mixtures with different mass fractions of GM powder has been shown before using cotton materials processed in the same way as described for the GHB119 cotton [11]. Here we report on the results of a homogeneity study performed on each of the two GM cotton mixtures. Additionally, the recommended minimum sample intake is discussed.

4.1 Homogeneity study

The degree of homogeneity of the powder in ERM-BF428b and c with respect to the mass fraction of GHB119 cotton was measured by real-time PCR using a random stratified procedure. This homogeneity study was planned together with the measurements to control the gravimetric preparations (Section 3.5). As the measurement results were obtained under repeatability conditions on bottles randomly taken from the entire batch and analysed in a randomised order they were as well suited to investigate the homogeneity. For ERM-BF428b and c data obtained on 2 subsamples from 15 random selected bottles ($N = 15$, $n = 2$) were available. Each subsample was measured in three replicates by real-time PCR.

In a first step it was checked whether the data followed a normal distribution using normal probability plots and histograms. The individual data and the bottle averages for the homogeneity data measured for ERM-BF428c were normally distributed. No outliers were detected for these data applying the Grubbs tests. A regression analysis was used to evaluate potential drifts in results related to the analysis sequence or to the filling sequence. No significant trends were observed in the results.

ANOVA statistics were used to calculate the between bottle standard deviation (s_{bb}) and the relative maximum standard uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), using the formulas [12]:

$$s_{bb} = \sqrt{\frac{MS_{bb} - MS_{wb}}{n}} \qquad u^*_{bb} = \sqrt{\frac{MS_{wb}}{n}} \cdot \sqrt[4]{\frac{2}{df_{wb}}}$$

(MS_{bb} = mean sum of squares between bottles; MS_{wb} = mean sum of squares within bottles; n = number of replicates; df_{wb} = degrees of freedom within bottles)

Both values were converted into relative uncertainties and were expressed in percentage (Table 4).

The data obtained for ERM-BF428b proved to be not normally distributed and followed a bimodal distribution. For this reason u^*_{bb} was calculated using an approach described in [14] and resulting in the following formula:

$$u^*_{bb} = \frac{(v_{max} - v_{min})}{2\sqrt{3}}$$

(v_{max} = highest value measured (11.8 g/kg); v_{min} = lowest value measured (6.7 g/kg)

The resulting value (1.47 g/kg) was converted into a relative uncertainty ($u^*_{bb,\,rel}$), expressed in percentage. The results of the homogeneity testing are summarised in Table 4.

The $u^*_{bb,\,rel}$ values were included into the calculation of the overall uncertainty on the certified values (Section 7.2).

Table 4: Relative standard uncertainties linked to the heterogeneity between bottles of dry-mixed GHB119 cotton candidate CRMs analysed by real-time PCR

Candidate CRM	Relative between bottle heterogeneity ($s_{bb,\,rel}$) [%]	Relative maximum hidden heterogeneity ($u^*_{bb,\,rel}$) [%]
ERM-BF428b	n.a.	14.7
ERM-BF428c	- [1)]	2.9

[1)] As MS_{bb} was smaller than MS_{wb}, s_{bb} could not be calculated.

4.2 Minimum sample intake for analysis

The cotton DNA extraction method employed here recommends a sample intake of 1 g and mentions the possibility to use 500 mg [9]. Within the frame of the gravimetric control and homogeneity measurements (Section 3.5 and 4.1), it was shown that the CTAB extraction method in combination with Genomic Tip 20 leads to reliable PCR results also if 500 mg sample intake is used. Therefore, the minimum amount of sample to be used is 500 mg.

5 Stability

5.1 Short-term stability

The short-term stability of ERM-BF428 was investigated following isochronous incubation of bottles at 4, 18 and 60 °C for 1, 2 and 4 weeks. ERM-BF428c was chosen for this study as it contains the highest mass fraction of GHB119 of the three cotton candidate CRMs (nominal 100 g/kg). From each of the 5 bottles per condition (N = 5), two subsamples (n = 2), were analysed for stability of the DNA in the matrix. A similar number of reference samples were likewise analysed, which were kept at the reference temperature of -70 °C during the 4 weeks. The same DNA extraction and real-time PCR method was used as for the verification of the mass fraction (Section 3.5) and the homogeneity (Section 4.1).

Scrutinising the data obtained, the single and double Grubbs tests revealed that the data sets for each temperature contain one outlier at 95 % confidence level. As no technical reasons for the outliers could be identified the outlying data were retained. Regression analysis revealed no trend over the time period of 4 weeks for the samples incubated at 4, 18 and 60 °C (t-test, 95 % confidence level). Based on these results and the existing experience about the stability of cotton seed powders, it was concluded that the uncertainty due to degradation during dispatch is negligible for all three candidate CRMs. ERM-BF428 can be shipped under ambient conditions.

5.2 Long-term stability

The long-term stability of a similar produced cotton seed powder CRM (ERM-BF422b) has been investigated earlier. An isochronous incubation of bottles at 4 and 18 °C for 3, 6 and 12 months was carried out. As ERM-BF422b is a ground cotton seed powder certified for the mass fraction of the cotton event 281-24-236 and the event 3006-210-23, the stability of both was investigated. Accepting a shelf life of 12 months, the highest relative standard uncertainty contribution ($u_{lts,\ rel}$) for the stability was 4.6 %, which was later used as an estimation of the standard uncertainty contribution for the stability of the CRM ERM-BF428 (Section 7.2).

It is generally recommended to store bottles of ERM-BF428 in the dark and within the temperature interval of (4 ± 3) °C.

6 Characterisation

The three candidate CRMs under the label ERM-BF428 are cotton seed powder materials processed from non-GM and GM seeds. While ERM-BF428a is prepared from the pure blank material, the other CRMs of the ERM-BF428 series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF428 is being certified for its GHB119 mass fraction.

The mass values are based on the mass fractions of dry-mixed GM and non-GM powder, corrected for their water mass fractions, and taking into account the powder's purity with regard to the GHB119 event. The values were calculated according to the following formulas:

$$\text{Mass fraction of GM material [g/kg]} = \frac{m_{\text{GM,anhyd}}\,[\text{g}] \times p_{\text{GM}}\,[\text{g/g}]}{m_{\text{GM,anhyd}}\,[\text{g}] + m_{\text{nonGM,anhyd}}\,[\text{g}]} \times 1000$$

$$m_{\text{GM,anhyd}}\,[\text{g}] = m_{\text{GM}}\,[\text{g}] \times (1 - \text{WMF}_{\text{GM}}\,[\text{g/g}])$$

$$m_{\text{nonGM,anhyd}}\,[\text{g}] = m_{\text{nonGM}}\,[\text{g}] \times (1 - \text{WMF}_{\text{nonGM}}\,[\text{g/g}])$$

(anhyd = anhydrous; p_{GM} = purity of the GM powder used for the dilution; WMF = water mass fraction)

In Table 5, the data supporting the calculation of the mass fractions of GHB119 are summarised.

Table 5: Subsequent mixing of GM GHB119 cotton seed powder with non-GM powder with to prepare ERM-BF428 materials

ERM code	GM powder			Non-GM powder[1)]	Mixtures
	Mass fraction of GM powder [g/kg]	Water mass fraction ± *U* (*k* = 2) [g/kg]	Mass [g]	Mass [g]	Resulting mass fraction of GM powder [g/kg]
BF428c	1000.0	17.4 ± 2.5	399.4	3600.6	100.0
BF428b	100.0 [2)]	21.2 ± 3.0	400.8	3599.2	10.0
BF428a	n.a.	n.a.	0	4000.0	0.0

[1)] The non-GM powder used for the gravimetric preparations had a water mass fraction of 19.1 ± 2.7 g/kg (*U, k* = 2) and was considered to be free of GHB119 cotton.
[2)] For the preparation of BF428b, the 100 g/kg GM powder was used.

7 Certified values and uncertainty budgets

7.1 Certified value

ERM-BF428 is series of three CRMs certified for the mass fraction of GHB119 cotton seed powder. The certified values are based on the masses of dried powder of GM seeds and non-genetically modified 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 (Section 6).

Purity of the GM and non-GM batches used for the processing of these powders were investigated in order to be able to calculate the certified value. No indication was found that the GM GHB119 cotton seed base material contained seeds being negative for the event GHB119 (Section 3.1). No indication for the presence of GHB119 was found in the non-GM powder by real-time PCR (Table 3). As no evidence for a contamination was found in both base materials, 100 % purity was used for the calculation of the certified mass fraction of GHB119 in the powder mixtures.

The powder used for the production of ERM-BF428a did not contain traces of the GHB119 cotton above the LOD of the applied real-time PCR method (Section 3.5). The certified value for ERM-BF428a is therefore based on the LOD of the real-time PCR method applied, as determined during in-house method validation.

Real-time PCR measurements demonstrated that no mixing errors were made (Table 3). Gel electrophoresis proved that the DNA analyte was not degraded during processing of the CRM (Figure 3).

As 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, the user is reminded that IRMM only certifies these materials for their mass fraction of GHB119. The envisaged copy number ratio certification might reveal if DNA extraction differences exist. Additionally one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements on unknown samples as DNA- and/or protein-based quantification of GMOs may vary with the particular matrix and the cotton variety tested [13].

7.2 Uncertainty budget

The expanded uncertainty of the certified value (U_{CRM}) comprises standard uncertainty contributions from the characterisation, the inhomogeneity, and the stability:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2}$$

The individual uncertainty contributions are summarised in Table 6.

The uncertainty introduced by the inhomogeneity has been estimated as the relative maximum heterogeneity potentially hidden by the method repeatability (u^*_{bb}) as defined in Section 4.1.

The uncertainty contribution from the stability (u_{lts}) has been estimated on the basis of stability tests on a cotton seed CRM having the same characteristics as ERM-BF428 and was calculated for 12 months (Section 5.2).

The u_{char} on the certified mass fraction of GHB119 was composed of several contributions, i.e. the uncertainty on the mass determination ($u_{char,1}$), the uncertainty on the water mass fraction analysis ($u_{char,2}$), and the uncertainties on the purity determination of the non-GM

and GM base powders ($u_{char,3}$ and $u_{char,4}$). Based on a statistical analysis of the probability distribution to find a negative seed in the GM base material, it could be concluded that the purity was higher than 98.5 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value.

A coverage factor of 2 ($k = 2$) was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % [14].

For the blank material, the LOD of the method was used to describe the 95 % confidence interval on the certified mass fraction of GHB119 (< 0.2 g/kg). This is supported by the high purity of the (non-GM) material and the absence of any mixing step; calculating the U_{CRM} for the blank material on the basis of the only quantifiable standard uncertainty ($u_{char,3}$) resulted in a value of 0.12 g/kg, which is below the certified < 0.2 g/kg value. The LOD is, therefore, already a conservative estimate of the certified value and no uncertainty is assigned.

Table 6: Uncertainty budgets for the mass fractions of GHB119 cotton in ERM-BF428

ERM	Certified value [g/kg]	Standard uncertainty contribution [g/kg]						Expanded uncertainty [8] U (k = 2) [g/kg]
		u_{bb} [2]	u_{lts} [3]	$u_{char,1}$ [4]	$u_{char,2}$ [5]	$u_{char,3}$ [6]	$u_{char,4}$ [7]	
BF428a	**< 0.2** [1]	n.a.	n.a.	n.a.	n.a.	0.0577	n.a.	**-**
BF428b	**10.0**	1.4700	0.4600	0.0117	0.0006	0.0577	0.0432	**4**
BF428c	**100**	2.9000	4.6000	0.0825	0.0045	0.0577	0.4323	**11**

[1] With a 95 % probability, the certified value is below this level.
[2] Standard uncertainty contribution resulting from the homogeneity assessment.
[3] Standard uncertainty resulting from the stability study of dried cotton seed powders during storage at 4°C, extrapolated to 12 months.
[4] Standard uncertainty of the mass determination mainly based on the uncertainty of the balance and the number of weighing steps required.
[5] Standard uncertainty of the water mass fraction determination by volumetric KFT.
[6] Standard uncertainty of the purity estimation of the non-GM base material (LOD = 0.2 g/kg), based on the half-width of the interval between 0 and 0.2 g/kg, divided by the square root of 3 (rectangular distribution).
[7] Standard uncertainty of the purity estimation of the GM base material (> 98.5 %), based on the interval between 98.5 % and 100 % divided by the square root of 3 (rectangular distribution).
[8] Rounded expanded uncertainties are given.

8 Metrological traceability

The ERM-BF428 series is composed of three reference materials certified for the mass fraction of event GHB119 cotton seed powder. The certified values are based on gravimetric dry-mixing of non-modified cotton seed powder with event GHB119 cotton seed powder. The respective certified value is traceable to the International System of Units (SI). The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure. The purity of the used seeds has been taken into account when calculating the certified value.

9 Intended use and instructions for use

The ERM-BF428 series of CRMs is intended for use as quality control material or calibrant in DNA-based methods for the detection of genetically modified material in food and feed.

The minimum amount of sample to be used is 500 mg.

The materials are hygroscopic. Bottles should be stored dry and dark at (4 ± 3) °C. The user is advised to close bottles immediately after taking a sample for analysis.

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European Commission

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Abstract
This report describes the processing and certification of three cotton seed powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) GHB119 cotton (ERM-BF428a, b, c). The materials were processed and certified in 2008/2009 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), according to the principles of ISO Guide 34. The three CRMs have been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

GM seeds of GHB119 cotton and of a non-GM cotton variety were dried and ground to obtain GM and non-GM base powders. A non-GM pure material was prepared. Gravimetric mixtures of non-GM and GM cotton powder were prepared by dry-mixing, a first material by mixing non-GM and GM cotton powder and a second one by further dilution of the mixture with non-GM cotton powder. The certified values of these CRMs were calculated from the gravimetric preparations, taking into account the purity of the base materials and their water mass fraction. The certified values were confirmed by event-specific real-time PCR as independent verification method (measurements within the scope of accreditation to ISO/IEC 17025).

The certified values and uncertainties of the three CRMs are as follows:

CRM	Quantity [1)]	Certified value [g/kg]	Uncertainty [3)] [g/kg]
ERM-BF428a	Mass fraction	< 0.2 [2)]	-
ERM-BF428b	Mass fraction	10.0	4
ERM-BF428c	Mass fraction	100	11

[1)] Mass fraction of GHB119 cotton (unique identifier code BCS-GHØØ5-8) based on the masses of genetically modified GHB119 cotton seed powder and non-modified cotton seed powder and their respective water content. The certified value is traceable to International System of Units (SI).
[2)] With a 95 % probability, the value of the material is below this level.
[3)] The certified uncertainty is the expanded uncertainty (U) estimated in accordance with the Guide to the Expression of Uncertainty in Measurement with a coverage factor $k = 2$, corresponding to a level of confidence of about 95 %.

The CRMs are intended for the quality control or calibration of methods for the quantification of GHB119 cotton in food and feed. The CRMs are available in glass vials containing 1 g of dried cotton seed powder closed under argon atmosphere. The minimum amount of sample to be used is 500 mg.

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