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 T304-40

Certified Reference Materials ERM®-BF429 (ERM®-BF429a, ERM®-BF429b, ERM®-BF429c)

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European Commission
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Contact information
Reference materials sales
Retieseweg 111
B-2440 Geel, Belgium
E-mail: jrc-irmm-rm-sales@ec.europa.eu
Tel.: +32 (0)14 571 705
Fax: +32 (0)14 590 406

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

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Certification of Reference Materials of Cotton Seed Powder with Different Mass Fractions of the Cotton Event T304-40

Certified Reference Materials ERM®-BF429 (ERM®-BF429a, ERM®-BF429b, ERM®-BF429c)

S. Trapmann, P. Corbisier, A.M. Kortekaas,
M.F. Tumba-Tshilumba, S. Vincent

European Commission, Joint Research Centre
Institute for Reference Materials and Measurements (IRMM), Geel (BE)

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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) T304-40 cotton (ERM-BF429a, b, c). The materials were prepared and certified in 2009/2010 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 T304-40 cotton and of a non-GM cotton variety were washed and dried twice, afterwards grinding was applied 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 respective purity of the base materials and their water mass fractions. Quantification of the mass fraction of T304-40 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF429 (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-BF429a	Mass fraction	< 0.4 [2)]	-
ERM-BF429b	Mass fraction	10	1.3
ERM-BF429c	Mass fraction	100	11

[1)] Mass fraction of T304-40 cotton (unique identifier code BCS-GHØØ4-7) based on the masses of genetically modified T304-40 cotton seed powder and non-modified cotton seed powder and their respective water content. The certified value is traceable to the International System of Units (SI).
[2)] With 95 % confidence, the value of the material is below 0.4 g/kg.
[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 T304-40 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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GLOSSARY

ANOVA	analysis of variance
EuRL-GMFF	European Reference Laboratory for Genetically Modified Food and Feed
CRM	Certified Reference Material
Ct	Cycle threshold
CTAB	cethyl trimethyl ammonium bromide
DNA	deoxyribonucleic acid
ERM®	trademark of European Reference Materials
T304-40	GM cotton (*Gossypium hirsutum)* event T304-40
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
TE	tris hydroxymethyl aminomethane (TRIS) ethylene diamine tetraacetate (EDTA)
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 or 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 T304-40 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 T304-40. Following Commission Regulation (EC) No 65/2004 [3], the T304-40 event received the unique identifier code BCS-GHØØ4-7.

In 2008, the IRMM was asked by Bayer BioScience to develop and produce a series of reference materials for the quantification of T304-40 cotton. The resulting CRM has been named ERM-BF429 and is composed of a series of three CRMs with different mass fractions of the genetically modified T304-40 cotton seed. The user of ERM-BF429 (T304-40 cotton) should note that the intellectual property owner informed IRMM about his intention to apply for authorisation in Europe of a stacked cotton event T304-40 x GHB119.

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 T304-40 cotton seeds to IRMM. After arrival, the cotton seeds were stored (4 ± 3) °C in the dark until use. Thirty kg of non-GM cotton seeds and 10 kg of T304-40 cotton seeds were used for the processing of ERM-BF429.

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 T304-40. 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 European Reference Laboratory for GM food and feed [4]. Genomic DNA extracted from pure T304-40 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 T304-40 event. Statistical analysis (Poisson distribution for rare events) revealed that the GM cotton seed batch had a genetic purity of > 98.5 % (95 % level of confidence).

Table 1: Seed lot purity of the GM cotton seed batch used for the processing of ERM-BF429 with respect to GM event T304-40. The resulting lot purity is based on a Poisson distribution at a 95 % level of confidence and is expressed as number fraction.

Batch	Number of seeds tested	Number of T304-40 positives	Number of T304-40 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) for the mass fraction of 0.4 g/kg the method did not detect the event T304-40 (Section 3.5).

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 applying systematic cleaning 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 already cleaned from cotton fibres by an acid delinting treatment. This delinting treatment included a neutralisation of the acid by addition of NaOH. A thorough two step washing and drying procedure was therefore carried out at IRMM to remove the residues from the black seeds. The cotton seeds were rinsed for about 15 min with water, drained, and dried on special trays in a drying chamber at 30 °C (Elbanton, Kerkdriel, NL). The drying time was extended until the mass of the seeds before washing was diminished by about 3 %. The rinsing, draining and drying step was repeated and resulted in visually clean seeds. After these two washing and drying steps the non-GM seeds had a remaining residual water mass fraction of 76 g/kg with an expanded uncertainty of 11 g/kg (k = 2) and the GM seeds had a remaining residual water mass fraction of 79 g/kg with an expanded uncertainty of 12 g/kg (k = 2). The mass fraction of water was determined by volumetric Karl Fischer titration (KFT). The seeds were then milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). Prior to this milling step the cotton seeds were frozen overnight in approximately 5 kg portions in metal containers in liquid nitrogen. The mill was also cooled down to process the seeds at a temperature below -95 °C. The feeding speed of the mill was set in such a way as to ensure most efficient milling with respect to the particle size obtained. After milling, the powder was kept at room temperature to reach a thermal equilibrium. The GM and non-GM powders were then sieved separately with a 1000 µm stainless steel mesh on a Russel Finex (London, UK). In case of the GM powder all material passed the 1000 µm mesh. In case of the non-GM powder a coarse fraction did not pass the 1000 µm mesh and was therefore cryo-milled a second time under the same conditions as described before. After reaching thermal equilibrium at room temperature, the fraction milled for a second time was sieved with the same 1000 µm mesh as previously. The whole material passed the mesh. Each 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 and sieving process.

For the GM and non-GM powder a residual water mass fraction of 105 g/kg with an expanded uncertainty of 15 g/kg (k = 2) was measured. In order to reduce the water content the powders were dried over night under vacuum in a freeze drier (Epsilon 2-65D, Osterode, DE) at 30 °C. The particle volumes were measured based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and compared for both powders. Based on the difference in the particle volumes found it was decided to sieve the non-GM powder on a 500 µm stainless steel mesh and to mill the coarser fraction once more. About 5 kg of the approximately 25 kg material remained on the sieve and were milled. After reaching the thermal equilibrium the material was mixed again in a DynaMIX CM200 for 30 min and the particle volumes were measured once more and found acceptable (Figure 1). The average particle diameters (N = 1, n = 3), calculated by the PSA software, were 149 µm (s = 6 µm) for the GM powder and 194 µm (s = 13 µm) for the non-GM powder. Furthermore, the water mass fraction was measured once more and found suitable for dry-mixing. The final water mass fractions of the non-GM powder were 16.3 ± 2.3 g/kg (U, k = 2) and 9.1 ± 1.3 g/kg for the GM powder (Table 5).

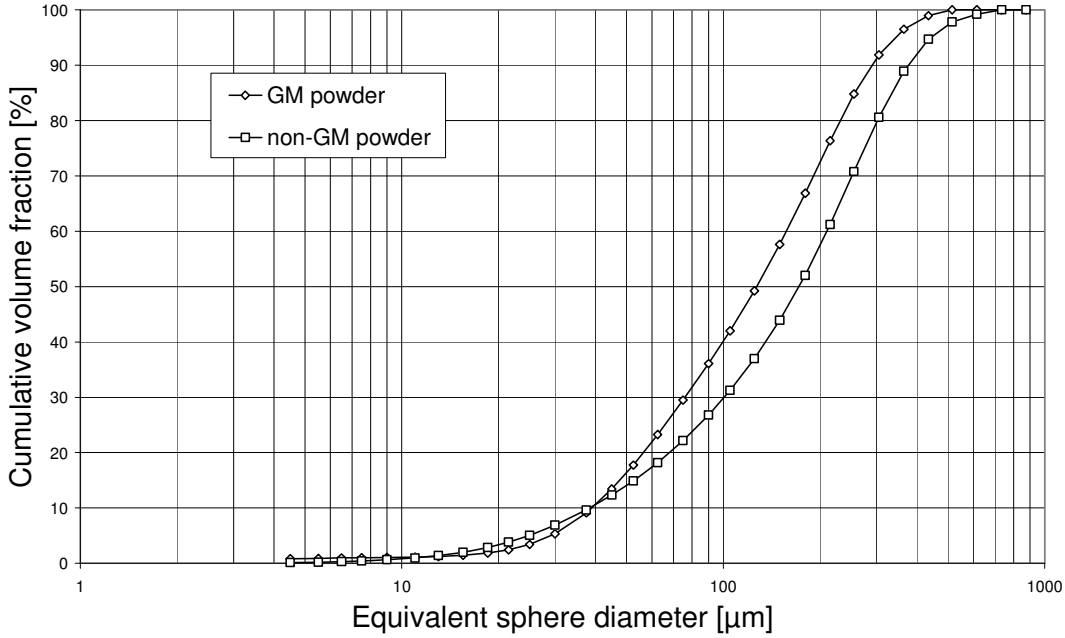

Figure 1: Accumulation of particle volume fractions in the GM powder and non-GM powder analysed by PSA. Each point represents an average of three replicates (*N* = 1, *n* = 3). The total volume is set as 100 %.

3.3 Gravimetric preparation of mixtures

The ground base materials were used to produce a blank material for T304-40 and two mixtures containing different mass fractions of T304-40 cotton seed powder in non-GM cotton seed powder at nominal mass fraction levels of 10 and 100 g/kg, respectively. 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 %. Weighed portions of the powder materials were placed 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 material having a nominal mass fraction of 100 g T304-40/kg was produced by mixing pure GM with pure non-GM ground base materials. The material having a nominal mass fraction of 10 g T304-40/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). Prior to the dry-mixing of 10 g T304-40/kg, a short additional freeze drying of about 6 h of the 100 g T304-40/kg material was required to further reduce the water content. 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 necks. 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 mass fraction levels of T304-40: nominal 0 g/kg = silver (BF429a), nominal 10 g/kg = red (BF429b), nominal 100 g/kg = brown (BF429c), consistent with the cap colours of previous IRMM CRMs for GMOs. Each of the vials was identified by a numbered label indicating the ERM code (Figure 2). 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 2: Prototype labels for the ERM-BF429 series. The denotation 'blank' was used for the nominal mass fraction of 0 g/kg T304-40 cotton powder (BF429a), while BF429b and BF429c refer to the nominal 10 g/kg and 100 g/kg T304-40 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-BF429 determined by volumetric KFT (N = 10, n = 1)

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	U (k = 2)
ERM-BF429a	18.5	2.5
ERM-BF429b	16.6	2.2
ERM-BF429c	14.0	1.9

Five randomly selected bottles from each of the powder materials were analysed twice for their particle volume distribution (N = 5, n = 2) 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 calculated volume of the particles based on their diameter is, therefore, overestimating the average particle size. The powders had a maximum particle diameter below 735 µm (Figure 3). The average particle diameters (N = 5, n = 2), calculated by the PSA software, were 177 µm (s = 22 µm), 184 µm (s = 11 µm) and 180 µm (s = 11 µm) for ERM-BF429a, 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].

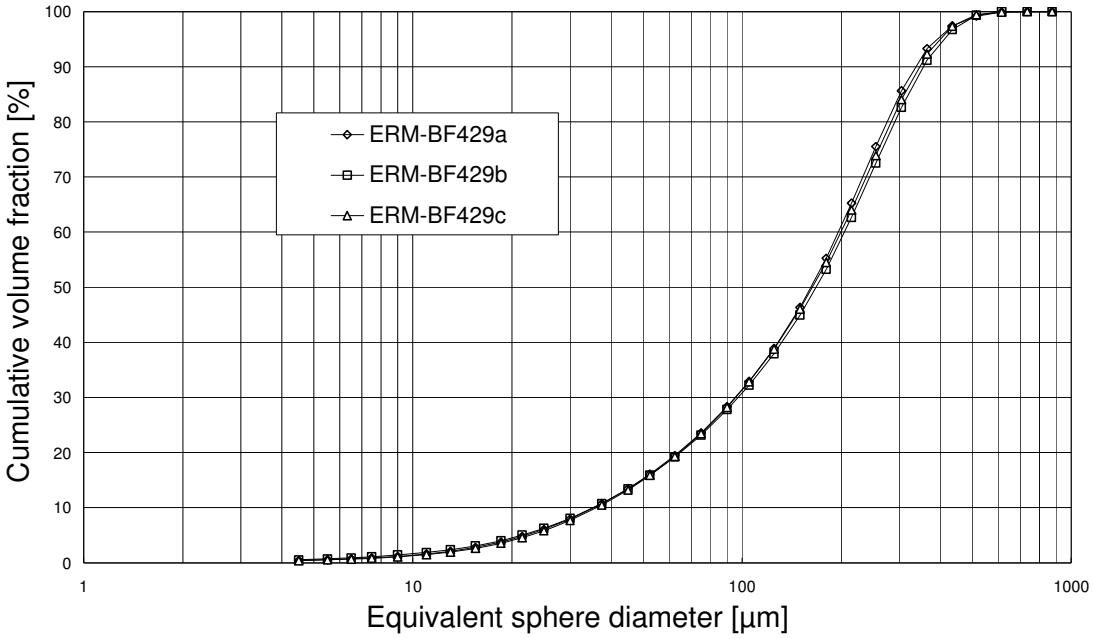

Figure 3: Accumulation of particle volume fractions in ERM-BF429 analysed by PSA (*N* = 5, *n* = 2). Each point represents an average of three replicate measurements (*N* = 1, *n* = 3). The total volume is set as 100 %.

Two 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. real-time PCR. Unfortunately, the classical fractionation method developed by Ogur and Rosen [7] does not allow the quantification of the DNA mass fraction. DNA spiking experiments for cotton matrices 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]. 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.

The DNA integrity was checked by gel electrophoresis. From two subsamples of each of the processed powder materials for ERM-BF429a, BF429b and BF429c, DNA was extracted from 500 mg powder using a CTAB/Genomic DNA Tip 20 DNA extraction method validated by the EuRL GMFF for cotton seed [9]. Approximately 0.4 µg DNA was made visible on an agarose gel by UV after ethidium bromide staining with a mass concentration of 7.5 g/L. A 1 kb DNA ladder (New England Biolabs, Inc., Ipswich, MA, USA) was used to estimate the DNA size. None of the samples showed DNA degradation (Figure 4).

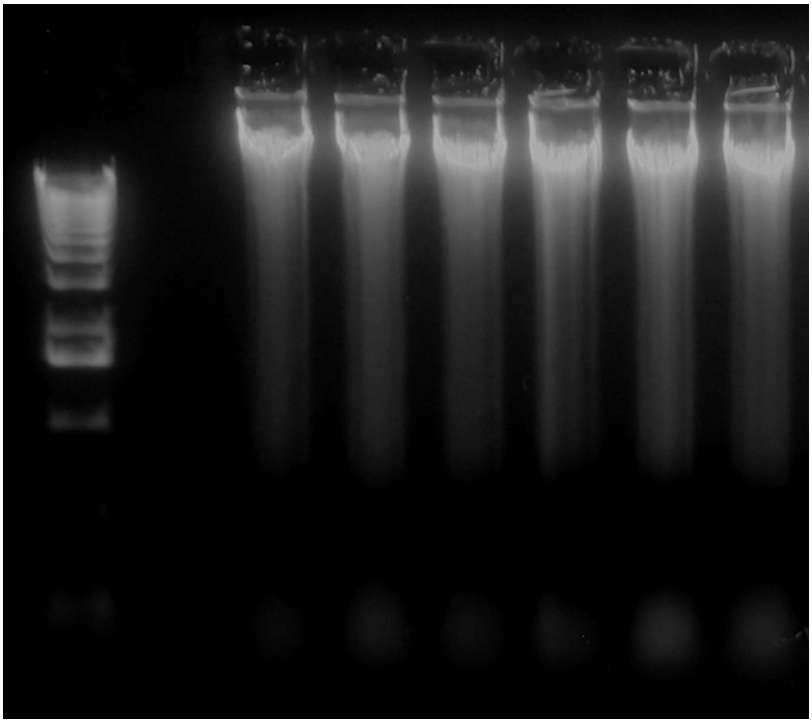

Figure 4: Agarose gel electrophoresis of DNA extracted from ERM-BF429a, b and c powder materials. The extraction method use was CTAB/Genomic DNA Tip 20 (*N* = 1, *n* = 2).

As a control for the gravimetric preparations, the mass fraction of T304-40 cotton in all three CRMs was verified by the confidential real-time PCR method provided by Bayer BioScience 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 EuRL GMFF [9]. The real-time PCR test was calibrated with genomic DNA extracted from the pure T304-40 cotton powder, and afterwards diluted in a TE buffer solution (pH 8.0, 1 mmol/L TRIS and 0.01 mmol/L EDTA) to produce a calibration curve between 4-times diluted and 2000-times diluted and between undiluted and 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 T304-40 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] but estimating *s* on the lowest dilution measured. The results of the quantification of T304-40 for the three candidate CRMs are shown in Table 3. Quantification of the mass fraction of T304-40 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF429. 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. It has to be noted that the calibrant used for the transgenic and the taxon-specific target is genomic DNA extracted from the pure T304-40 cotton powder, consequently the deviation from the nominal mass fraction values (10 and 100 g/kg) refers to a different extractability pattern or analytical behaviour of the taxon-specific target extracted from the non-GMO material or matrix effects caused by the GMO or the non-GMO material. However, the deviation can also be caused by more than one of these four effects acting simultaneously.

Table 3: Quantification of the T304-40 cotton mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure T304-40 seed powder for calibration

Candidate CRM	T304-40 cotton mass fraction [g/kg]	U (k = 2) [g/kg]
ERM-BF429a	< 0.4 [1)]	-
ERM-BF429b	13.5 [2)]	1.6
ERM-BF429c	129.6 [2)]	8.2

[1)] Average for 2 subsamples from each of 5 random bottles (N = 5, n = 2), with each subsample measured in three replicates. The obtained value is below the LOD determined during method validation (0.4 g/kg).
[2)] Average for 2 subsamples from each of 16 random bottles (N = 16, n = 2), with each subsample measured in three replicates.

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 T304-40 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-BF429b and c with respect to the mass fraction of T304-40 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-BF429b and c data obtained on 2 subsamples from each of 16 random selected bottles ($N = 16$, $n = 2$) 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-BF429b were normally distributed. The data measured for ERM-BF429c were unimodal 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 as percentage (Table 4). The $s_{bb,\,rel}$ value for ERM-BF429b and the $u^*_{bb,\,\mathbf{rel}}$ value for ERM-BF429c 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 T304-40 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-BF429b	4.0	3.0
ERM-BF429c	- [1)]	2.6

[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-BF429 was investigated following isochronous incubation of bottles at 4, 18 and 60 °C for 1, 2 and 4 weeks. ERM-BF429c was chosen for this study as it contains the highest mass fraction of T304-40 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 Grubbs tests revealed that the data set for the reference temperature contains one outlier at 95 % level of confidence. As no technical reasons for the outlier could be identified the outlying data was 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 % level of confidence). In order to be sure that the detected outlier in the reference data set does not lead to hiding an instability of the material at the temperatures of 4, 18 or 60 °C it was removed and the data sets reanalysed. Even under these conditions no trends were observed (t-test, 95 % level of confidence). 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-BF429 can be shipped under ambient conditions.

5.2 Long-term stability

The long-term stability of a similarly 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-BF429 (Section 7.2).

An intensive post certification monitoring is carried out to check the stability of ERM-BF429 at regular intervals of 1 year.

It is generally recommended to store bottles of ERM-BF429 in the dark and within the temperature interval of (4 ± 3) °C.

6 Characterisation

The three candidate CRMs under the label ERM-BF429 are cotton seed powder materials processed from non-GM and GM seeds. While ERM-BF429a is prepared from the pure blank material, the other CRMs of the ERM-BF429 series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF429 is being certified for its T304-40 mass fraction.

The mass values are based on the mass fractions of dry-mixed GM and non-GM powders, corrected for their water mass fractions, and taking into account the powder's purity with regard to the T304-40 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 T304-40 are summarised.

Table 5: Subsequent mixing of GM T304-40 cotton seed powder with non-GM powder to prepare ERM-BF429 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]
BF429c	1000.0	9.1 ± 1.3	397.6	3602.4	100.0
BF429b	100.0 [2)]	9.1 ± 1.3	397.4	3602.6	10.0
BF429a	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 16.3 ± 2.3 g/kg (*U, k* = 2) and was considered to be free of T304-40 cotton.
[2)] For the preparation of BF429b, the 100 g/kg GM powder was used.

7 Certified values and uncertainty budgets

7.1 Certified value

The ERM-BF429 series is composed of three CRMs certified for the mass fraction of T304-40 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 T304-40 cotton seed base material contained seeds being negative for the event T304-40 (Section 3.1). No indication for the presence of T304-40 was found in the non-GM powder by real-time PCR (Table 3). As no evidence for a contamination was found in either of the base materials, 100 % purity was used for the calculation of the certified mass fraction of T304-40 in the powder mixtures.

The powder used for the production of ERM-BF429a did not contain traces of the T304-40 cotton above the LOD of the applied real-time PCR method (Section 3.5). The certified value for ERM-BF429a is therefore based on the LOD of the real-time PCR method applied, as determined during in-house method validation at IRMM.

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 4).

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 T304-40. 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 ($u^*_{bb,\ rel}$) or the relative between bottle heterogeneity ($s_{bb,\ rel}$) as defined in Section 4.1.

The uncertainty contribution from the stability (u_{lts}) has been estimated on the basis of stability tests on another cotton seed CRM having similar physical properties as ERM-BF429 and was calculated for 12 months (Section 5.2).

The u_{char} on the certified mass fraction of T304-40 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 % level of confidence, Section 3.1). This 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 % level of confidence on the certified mass fraction of T304-40 (< 0.4 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 $U = 0.23$ g/kg, which is below the certified < 0.4 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 T304-40 cotton in ERM-BF429

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)	
BF429a	**< 0.4** [1]	n.a.	n.a.	n.a.	n.a.	0.1155	n.a.	**-**
BF429b	**10.0**	0.4002	0.4603	0.0124	0.0032	0.1155	0.0433	**1.3**
BF429c	**100**	2.6014	4.6025	0.0875	0.0265	0.1155	0.4325	**11**

[1] With 95 % confidence, the certified value is below 0.4 g/kg.
[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 = 0.4 g/kg), based on the half-width of the interval between 0 and 0.4 g/kg, divided by the square root of 3 (rectangular distribution).
[7] Standard uncertainty of the purity estimation of the GM base material, 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 and commutability

8.1 Metrological traceability

The ERM-BF429 series is composed of three reference materials certified for the mass fraction of event T304-40 cotton seed powder. The certified values are based on gravimetric dry-mixing of non-modified cotton seed powder with event T304-40 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 and the water content of the used seeds has been taken into account when calculating the certified value.

8.2 Commutability

ERM-BF429 is prepared from non-GM and GM cotton seed powder. DNA extracted from ERM-BF429 and targeted by the event-specific PCR method is genomic DNA. The type of DNA is therefore identical to the DNA extracted from food and feed samples and targeted by PCR. Provided a suitable DNA extraction and purification methods is chosen, which is leading to a genomic DNA quality and purity suitable for PCR amplification, commutability [15] problems can be excluded.

9 Intended use and instructions for use

The ERM-BF429 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.

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

The user of ERM-BF429 (T304-40 cotton) should note that the intellectual property owner informed IRMM about his intention to apply for authorisation in Europe for a stacked cotton event T304-40 x GHB119. According to Bayer BioScience the stacked event will have a ratio of the transgenes T304-40 to GHB119 to the endogene suggested for quantification of the event of 1:1:1. Reference material for the event GHB119 cotton is available under the code ERM-BF428 [16].

References

[1] European Commission (2003) Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. Off. J. Eur. Union, L 268:24-28.

[2] Commission Recommendation (EC) No 787/2004 of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003, Official Journal of the European Union (2004), L 348: 18-26.

[3] European Commission (2004) Commission Regulation (EC) No 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms. Off. J. Eur. Union, L10:5-10.

[4] European Reference Laboratory for GM food and feed (EuRL GMFF) Status of dossiers http://GM-crl.jrc.ec.europa.eu/statusofdoss.htm.

[5] TaqMan® Universal PCR Master Mix protocol, Applied Biosystems. See website http://docs.appliedbiosystems.com/pebiodocs/04304449.pdf.

[6] Moreano F., Busch U., Engel K. H. (2005) Distortion of genetically modified organism quantification in processed foods: influence of particle size compositions and heat-induced DNA degradation. J. Agric. Food Chem. 28:9971-9979.

[7] Ogur M., Rosen G. (1950) The Nucleic Acids of Plant Tissues. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. 25:262-276.

[8] Ganguli P.K. (1970) A sensitive procedure for the estimation of deoxyribonucleic acid by the diphenylamine reaction in the presence of cupric sulphate. Rev. Can. Biol. 29: 339-346.

[9] Community Reference Laboratory for GM food and feed (2009) Sampling and DNA extraction of cotton seeds - Report from the Validation of the "CTAB/Genomic-tip 20" method for DNA extraction from ground cotton seeds http://GM-crl.jrc.ec.europa.eu/summaries/281-3006%20Cotton_DNAExtr.pdf.

[10] DIN 32645 (2008) Chemische Analytik. Nachweis-, Erfassungs- und Bestimmungsgrenze. Beuth, Berlin.

[11] Trapmann S., Broeders S., Charoud-Got J., Conneely P., Contreras M., Corbisier P., Gioria S., Oostra A., Schimmel H., Van Nyen M., Emons H. (2006): The Certification of Reference Materials of Cotton Seed Powder with different Mass Fractions of the Cotton Event 281-24-236 x 3006-210-23, Certified Reference Materials ERM®-BF422, EC certification report EUR 22527 EN, ISBN: 92-79-03734-X.

[12] Linsinger T.P.J., Pauwels J.,van der Veen A.M.H., Schimmel H., Lamberty A. (2005) Homogeneity and stability of reference materials. Accred. Qual. Assur. 6:20-25.

[13] Trapmann S. (2006) Application Note 4: Use of Certified Reference Materials for the quantification of GM in food and feed. See http://www.erm-crm.org/html/ERM_products/application_notes/application_note_4/index.htm.

[14] EURACHEM/CITAC Guide (2000) Quantifying Uncertainty in Analytical Measurement. See http://www.measurementuncertainty.org/mu/guide/index.html.

[15] International vocabulary of metrology – Basic and general concepts and associated terms (VIM), JCGM 200:2008, http://www.bipm.org/utils/common/documents/jcgm/JCGM_200_2008.pdf

[16] Trapmann S., Charoud-Got J., Corbisier P., Jeynov B., Kortekaas A.M., Merveillie A., Vincent S. (2009) Certification of reference materials of cotton seed powder with different mass fractions of the cotton event GHB119 - Certified Reference Material ERM®-BF428, EC certification report EUR 24110 EN, ISBN 97892-79-14553-7.

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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) T304-40 cotton (ERM-BF429a, b, c). The materials were prepared and certified in 2009/2010 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 T304-40 cotton and of a non-GM cotton variety were washed and dried twice, afterwards grinding was applied 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 respective purity of the base materials and their water mass fractions. Quantification of the mass fraction of T304-40 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF429 (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-BF429a	Mass fraction	< 0.4 [2)]	-
ERM-BF429b	Mass fraction	10	1.3
ERM-BF429c	Mass fraction	100	11

[1)] Mass fraction of T304-40 cotton (unique identifier code BCS-GHØØ4-7) based on the masses of genetically modified T304-40 cotton seed powder and non-modified cotton seed powder and their respective water content. The certified value is traceable to the International System of Units (SI).
[2)] With 95 % confidence, the value of the material is below 0.4 g/kg.
[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 T304-40 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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