Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of reference materials of maize seed powder with different mass fractions of genetically modified 98140 maize

Certified Reference Material ERM®-BF427 (ERM®-BF427a, ERM®-BF427b, ERM®-BF427c, ERM®-BF427d)

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The mission of the IRMM is to promote a common and reliable European measurement system in support of EU policies.

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Certified Reference Material ERM-BF427 (ERM®-BF427a, ERM®-BF427b, ERM®-BF427c, ERM®-BF427d)

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GLOSSARY

als	acetolactate synthase encoding gene
ANOVA	Analysis of Variance
b	slope in the equation of the linear regression $y = bx + a$
CRM	Certified Reference Material
CRL	Community Reference Laboratory
Ct-value	number of PCR cycles to pass a set threshold
df_{wb}	degrees of freedom within bottle
DNA	deoxyribonucleic acid
ERM®	European Reference Material
ELISA	Enzyme-Linked Immuno Sorbent Assay
*gat*4621	gene coding for an glyphosate acetyltransferase (GAT) protein and conferring resistance to the herbicide glyphosate
gDNA	genomic DNA
GM	genetically modified
GMO	genetically modified organism
hra	gene coding for the acetolactate synthase (ALS) protein conferring tolerance to ALS-inhibiting herbicides
IRMM	Institute for Reference Materials and Measurements
JRC	Joint Research Centre
k	coverage factor
KFT	Karl Fischer titration
hmg	maize-specific reference gene used for GMO quantification, i.e. the single-copy *Zea Mays* high mobility group gene
LOD	limit of detection
LOQ	limit of quantification
MS_{bb}	mean sum of squares between bottles
MS_{wb}	mean sum of squares within bottle
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
rt-PCR	real-time PCR
s	standard deviation
s_{bb}	standard deviation between bottles
$s_{bb,\,rel}$	relative standard deviation between bottles
SI	International System of Units
U	expanded uncertainty
u^*_{bb}	standard uncertainty component due to the heterogeneity that can be hidden by method repeatability
$u^*_{bb,\,rel}$	relative standard uncertainty component due to the heterogeneity that can be hidden by method repeatability
u_{char}	standard uncertainty component due to the characterization of the material
u_{lts}	standard uncertainty component due to the long-term stability of the material
$\bar{x}$	average

1 Introduction

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] that are authorised in accordance with Community legislation. In general, this demands on the one hand, the development and validation of reliable GMO quantification procedures, and on the other hand, the production of reference materials for the quality control and calibration of these procedures.

According to regulation (EC) No 65/2004 [2] the event 98140 maize corresponds to the unique identifier DP-Ø9814Ø-6. The maize is genetically engineered to contain two additional genes: the *gat*4621 gene isolated from *Bacillus licheniformis* that confers tolerance to glyphosate herbicide and the *Zea mays hra* gene produced by modifying the *Zea mays als* gene that provides tolerance to acetolactate synthase-inhibiting herbicides.

The Institute for Reference Materials and Measurements (IRMM, Geel, BE) was asked by Pioneer Hi-Bred International Inc. (Johnston, IA, USA) to develop and produce a reference material for the quantification of 98140 maize. The major objective of the project was, therefore, the production of certified reference materials (CRMs) containing different mass fractions of the genetically modified (GM) 98140 maize seeds.

2 List of participants

Processing, characterisation and stability studies
European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel (BE), accred. ISO Guide 34 and ISO/IEC 17025, BELAC 268-TEST.

Homogeneity studies
Livsmedelsverket, Uppsala (SE), accred. ISO/IEC 17025, SWEDAC 1457.

3 Processing

3.1 Characterisation of the base materials

For the preparation of the CRMs, Pioneer Hi-Bred International Inc. (Johnston, IA, USA) supplied seeds of non-GM maize and GM maize to IRMM. Fifty kilogram of non-GM maize and 25 kg of GM maize were used for the processing of ERM®-BF427.

Pioneer Hi-Bred International Inc. carried out quality controls to assess the purity of the GM seed batch by testing 200 randomly chosen seeds for the presence of the transgene construct using an event-specific qualitative PCR assay specifically designed for the detection of the 98140 maize. The purity of the non-GM seed batch was not tested by Pioneer Hi-Bred International as it dated back from 1991 and has been produced before the development of the 98140 maize.

The purity of these batches was analysed at IRMM using genomic DNA (gDNA) extracted from leaves of seedlings in order to avoid influences from attached dust particles on the analytical results. Seeds of the non-GM and GM batches (N = 52 for each seed batch) were randomly chosen and allowed to germinate. Genomic DNA was extracted from pieces of the young leaves with a mass of approximately 100 mg for each plant using the QIAGEN Tissue Lyser® and DNeasy® Plant Mini kit (Qiagen, Hilden, DE).
The extracted DNA was quantified using the PicoGreen® DNA quantification kit [3]. The quantitative rt-PCR was performed at IRMM using an ABI 7900HT instrument and primer pairs and labelled TaqMan® probes specific for the 98140 event or for the maize reference gene encoding the high mobility group gene *hmg* [4], following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [5].

The results, summarised in Table 1, showed that all 52 plants analyzed from the GM batch contained the 98140 event. Similarly, all plants from the non-GM batch had a GM mass fraction below the limit of detection (0.4 g/kg, see below). Pioneer's results for the GM batch purity statement (purity of at least 98.5 %) were cross-checked using the Poisson distribution for rare events on results obtained by IRMM and revealed that the GM maize seed batch had a purity > 94 % (95 % confidence level).

Table 1: Purity of the GM and non-GM seed batches used for the processing of ERM-BF427 with respect to GM genes

Batch	Test results reported by	Number of seeds tested	Number of GM positive	Number of GM negative
Non-GMO	IRMM	52	0	52
GMO	IRMM	52	52	0
	Pioneer	200	200	0

The purity of the ground non-GM base material (certified as ERM-BF427a) expressed as mass fraction of GM contamination was tested at IRMM. The analysis of the powder from randomly selected vials (DNA extractions from three samples of 100 or 200 mg powder using the CTAB DNA extraction method, N = 5, n = 3) indicated that no detectable GM contamination was found in the non-GM lot, i.e. the values were all below the limit of detection (LOD) of 0.4 g/kg of the rt-PCR method applied (see Section 3.6).

Within the frame of an in-house validation of the method, the LOD and the limit of quantification (LOQ) were assessed. The LOD was calculated as $(3.3s)/b$, with s representing the standard deviation of the lowest GM mass fraction analysed and b the slope of the calibration curve. The efficiency of the amplification was determined based on

the slope of the regression line between the GM mass fraction and the *Ct*-values, which should be around the theoretical value of 3.322. The LOQ was calculated as (10*s*)/*b*.
LOD and LOQ have been established by dilution of DNA extracted from pure GM 98140 maize powder in nuclease-free water and were found to be 0.4 g/kg and 1.2 g/kg, respectively. This approach is also described in detail in DIN 32645 [6].

In conclusion, the analysis performed on the two seed powders (GM and non-GM) corroborated the Pioneer Hi-Bred Inc. purity statements.

3.2 Processing of the ground base materials

The GM and non-GM base materials were treated separately. Cross-contamination and contamination with foreign DNA were avoided using glove box systems and disposable laboratory clothing. All contact surfaces were treated with a DNA degrading solution prior to exposure to the base materials. An in-house validation study had proven beforehand, that the solution degrades DNA effectively under the given conditions.

The seeds to be processed were rinsed in demineralised water, drained, and dried under vacuum at 30 °C. This treatment led to a water mass fraction loss of approximately 35 g/kg in the case of the non-GM seeds and 18 g/kg in the case of the GM seeds. The dried seeds were then ground 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. An additional vacuum drying at 30 °C was carried out to further reduce the water content of the once ground base material by a mass fraction of approximately 70 g/kg. Then the dried-mixed powders were milled for a second time. The different fractions were then Dynamix-mixed altogether for 30-45 minutes. Care was also taken to avoid that the material was exposed to temperatures above 45 °C during mixing and milling. After measurement of the remaining water content, it was decided to carry out an additional vacuum drying at 30 °C. Finally, prior to the gravimetric preparation of the GM and non-GM mixtures by dry-mixing, the twice-ground base materials had a water mass fraction of approximately 10 g/kg for the non-GM powder and 12 g/kg for the GM powder (measured by volumetric Karl Fischer titration (KFT), Metrohm, Berchem, BE, $n = 3$).

3.3 Gravimetric preparation of GM mixtures

The twice-ground base materials were used to produce 4 kg of a GM blank material and of each of three powder mixtures containing mass fractions of 98140 maize at nominal levels of 5, 20 and 100 g/kg. Prior to the dry-mixing, the remaining mass fraction of water in the ground GM and non-GM base materials were determined in triplicate by volumetric KFT in order to correct the mass fractions for the water content of the ground base material. The mixture for the nominal mass fraction of 100 g/kg (ERM-BF427d) was produced first, by mixing GM with non-GM ground base material. All lower mass fractions were achieved by serial dilution of the 100 g/kg GM seed powder with non-GM maize seed powder. Ground base materials were weighed using a calibrated balance with a relative standard uncertainty lower than 0.1 %. The powders were first manually pre-mixed in a container, then Dynamix-mixed and finally mixed for 2 minutes in a dry-mixing device.

In Table 2, the data supporting the calculation of the certified values are summarised.

Table 2: Results from stepwise dilutions of ERM-BF427 based on gravimetry

ERM	GM powder			Non-GM powder [1)]	Resulting GM mass fraction
	Mass fraction [g/kg]	Water mass fraction [g/kg]	Mass [g]	Mass [g]	[g/kg]
BF427d [2)]	1000.0	12.0	400.6	3599.5	100.0
BF427c [2)]	100.0	12.5	801.3	3198.7	20.0
BF427b	20.0	10.0	999.6	3000.4	5.0

[1)] The non-GM powder used for the gravimetric preparations of the CRMs had a water mass fraction of 10.5 g/kg and was considered to be free of 98140 maize.
[2)] Due to container size limitations, these preparations were weighed and mixed in two batches, the two batches were afterwards merged.

3.4 Bottling

The dry-mixed powders were bottled as 1 g aliquots 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. Rubber stoppers were automatically placed on the vial neck. Before final closure of the vials, the air was evacuated in a freeze-drier and replaced by argon. The vials were closed with the help of a hydraulic device in the freeze-drier 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 GM levels: nominal mass fraction 0 g/kg - silver, 5 g/kg - blue, 20 g/kg - green and 100 g/kg - brown. For each mass fraction, labels were stuck onto each vial for identification (Figure 1). Following the inventory control and the selection of vials for future analysis according to a random stratified sampling scheme, the vials were brought to a storage room for long-term storage in the dark at 4 °C.

Figure 1: Lay-out of the labels for the four CRMs. ERM-BF427a label was used for the non-GM material (blank), and ERM-BF427b, ERM-BF427c, and ERM-BF427d labels were used for a respective nominal GM mass fraction of 5 g/kg, 20 g/kg and 100 g/kg.

3.5 Processing control

The residual mass fraction of water in the final batch of each CRM was determined by volumetric KFT in ten randomly selected bottles from each of the powder mixtures and gave values in the interval of 15 to 18 g/kg (Table 3).

Table 3: Water mass fraction of the four CRMs (*N* = 10, *n* = 1)

CRM	Water mass fraction [g/kg]	
	$\bar{x}$	*s*
ERM-BF427a	15.7	2.7
ERM-BF427b	15.9	5.5
ERM-BF427c	17.4	4.4
ERM-BF427d	15.7	2.6

Five randomly selected vials from each of the powder mixtures were used for particle size measurements with a particle size analyser based on laser diffraction (PSA, Sympatec, Clausthal-Zellerfeld, DE). From each vial, 2 subsamples were analysed. The powders had a maximum particle size below 870 µm (Figure 2) and an average particle size around 163 µm used for the calculation of the minimum sample intake (Section 4.2) and the calculation of the uncertainty budget (Section 6.2). Since most particles are presumably not perfectly spherical, the volume-based presentation of the PSA data is, therefore, overestimating the average particle size.

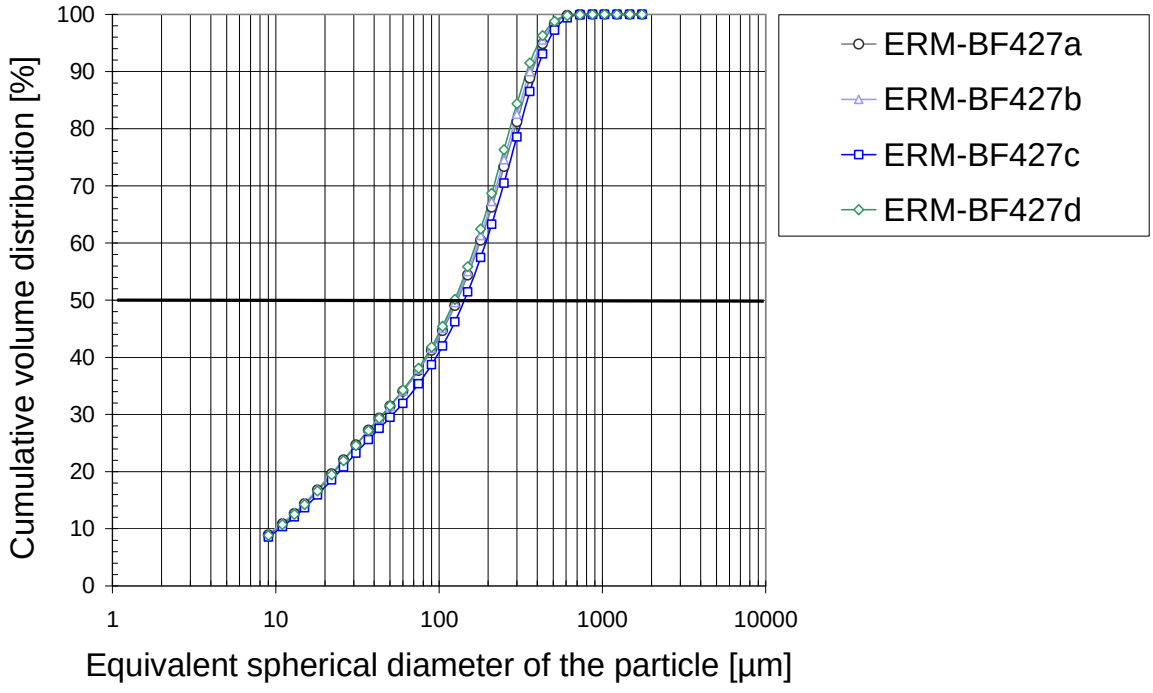

Figure 2: Average particle size distribution in ERM-BF427 by PSA (*N* = 5, *n* = 2). The cumulative volume distribution of particles derived from laser light scattering data (PSA) is based on their equivalent volume diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles.

Additionally, a sieving test was carried out following ISO 3310-1 [7] using sieves with meshes of 63, 90, 125, 180, 250, 500 and 710 µm (Figure 3) in order to confirm the data obtained with the particle size analysis. For sieving analysis the content of ten randomly selected bottles from each of the powder mixtures was merged to reach the required sample intake of 10 g. The maximum particle size of the materials was found to be smaller than 710 µm.

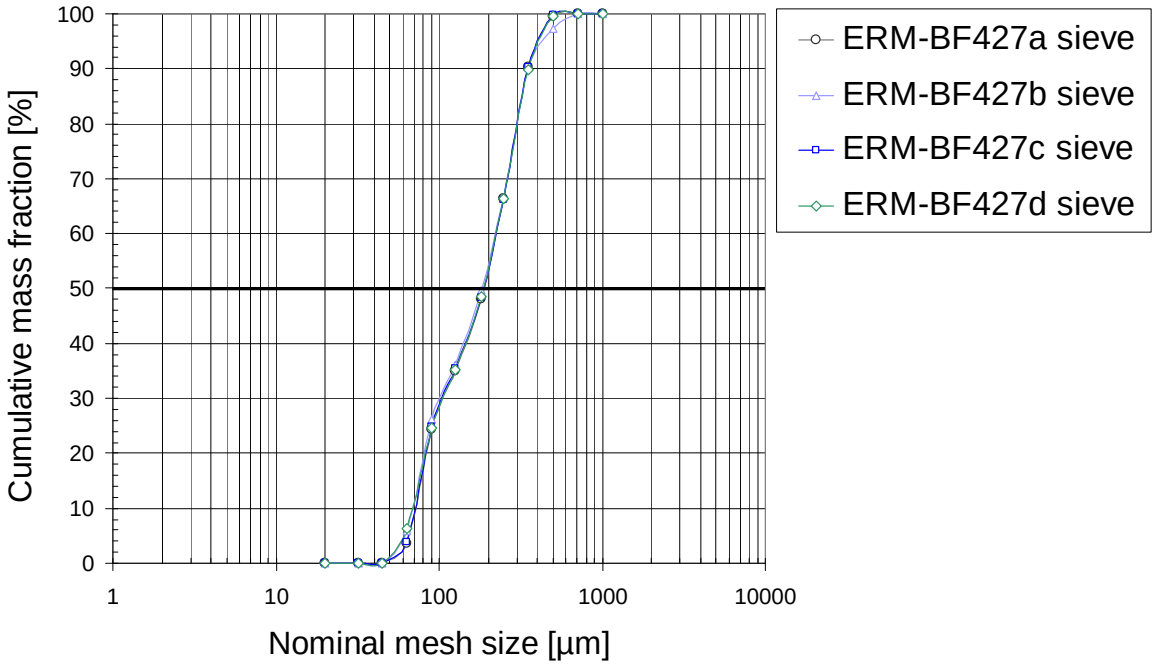

Figure 3: Average particle size distribution of ERM-BF427 by sieving analysis (*N* = 1). The cumulative mass fraction of the particles is derived from sieving analysis data.

Three of the described CRMs are mixtures of GM and non-GM maize seed powders, produced gravimetrically and certified for their GM powder mass fraction. The mass of DNA in both base materials was estimated by a slight modification of the classical fractionation method developed initially by Ogur & Rosen [8]. Indeed this quantity could influence the certified value of the GM and non-GM seed powders mixtures. A first step of removal of fatty compounds soluble in hexane was applied [9], followed by the sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds and acidic extraction at 70 °C with 0.84 mol/L perchloric acid pH 0.3. The mass of ethanol-precipitating DNA was measured spectrophotometrically after derivatisation with diphenylamine. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [8, 10]. The extractable DNA mass fraction of the two materials was calculated as:

$$\frac{\text{Extractable mass of DNA in 100 mg GM maize powder}}{\text{Extractable mass of DNA in 100 mg non - GM maize powder}}$$

The ratio of the DNA mass extractable from 100 mg of GM and non-GM maize seed powder was found to be 1.33 ± 0.04 (*N* = 9). A *t*-test demonstrated a significant difference between the DNA mass extracted from the GM and non-GM maize seed powders by the modified Ogur and Rosen method [8] (95 % confidence level). There is therefore evidence that the extractable DNA mass in the two powders differ. Consequently the GM mass fractions of ERM-BF427 prepared by gravimetry are not identical to the GM DNA copy number measured by rt-PCR. This difference can be attributed to the different origin and composition of the varieties between the GM and non-GM seeds as suggested by visual inspection.

3.6 Confirmation of maize mixtures

The GM mass fractions of all four CRMs were analysed using an event-specific rt-PCR method. During in-house validation, the LOD and LOQ of the method have been found to be 0.4 g/kg and 1.2 g/kg, respectively (for details see Section 3.1). The results obtained were corrected for the DNA mass difference found by the modified Ogur and Rosen method in proportion of the GM and non-GM mass of DNA present in the CRMs (Table 4).

Table 4: GM quantification in 98140 maize powders by event-specific real-time PCR. DNA was extracted from 100 or 200 mg powder sample intakes using the CTAB method. The measurements were calibrated with GM seed powder and data were corrected for the difference between the DNA mass extracted from the GM and non-GM seed powders.

CRM	98140 mass fraction determined by event-specific rt-PCR [1)] [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF427a	< 0.4 [2)]	n.a.
ERM-BF427b	4.9	0.8
ERM-BF427c	17.9	2.2
ERM-BF427d	96.4	9.9

[1)] For each CRM the content of five randomly selected bottles was analysed and five subsamples (N = 5, n = 5) of each were measured in three replicates.
[2)] The value was below the LOD of the method of 0.4 g/kg.

Quantification of the GM mass fraction in the three mixtures by rt-PCR did not reveal severe deviations from the gravimetrically prepared mass fractions of CRM ERM-BF427. However, one has to be careful to draw quantitative conclusions (in DNA copy number, for instance) from measurements of unknown samples as DNA-based GM quantification may vary with the particular matrix and maize variety tested [11].

4 Homogeneity

In order to ensure that the CRMs are sufficiently homogenous, two strategies were followed: validation of the mixing procedure and homogeneity control of the produced mixtures. The adequacy of the dry-mixing technology for the preparation of maize mixtures with different GM mass fractions has been shown previously using maize materials processed in the same way as described for the MON 863 maize [12].

Here we report on the results of a homogeneity study performed on each of the 98140 maize mixtures. Additionally, the recommended minimum sample intake is discussed.

4.1 Homogeneity study

The homogeneity of ERM-BF427 with respect to the 98140 maize mass fraction was investigated by rt-PCR using vials selected according to a random stratified procedure. The homogeneity of each of the CRMs, ERM-BF427b, ERM-BF427c and ERM-BF427d, was investigated using 15 to 18 vials that were analysed in random order under repeatability conditions using a sample intake of 150 mg powder and the CTAB DNA extraction method. The homogeneity measurements were performed by an external laboratory, selected for its previous experience in the field.

Grubbs tests were performed to detect outlying individual results as well as bottle averages. Two outliers for the double Grubbs test were detected for the ERM-BF424d material, but as no technical reason could be found to exclude them from the analysis, these results were retained.

Regression analyses were used to evaluate drifts in results related to the analysis sequence or to the filling sequence. No significant trends were observed in the results.

It was furthermore checked whether the data followed a normal or at least a unimodal distribution using normal probability plots and histograms respectively. The individual data and the bottle averages for all three CRMs were normally distributed.

ANOVA statistics were used to calculate the between bottles standard deviation (s_{bb}) and the maximum standard uncertainty related to the heterogeneity that can be hidden by the method repeatability (u^*_{bb}), using the formulas [13]:

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

(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 of s_{bb} and u^*_{bb} were converted into relative standard uncertainties and were expressed in percentage (Table 5). The largest of both values was included into the calculation of the overall uncertainty on the certified values (Section 6.2).

Comparison of the experimental data obtained during this homogeneity testing confirmed that the approach chosen for the estimation of the heterogeneity uncertainty contribution (Section 6.2, Table 6) was valid.

Table 5: Relative standard uncertainties due to heterogeneity between bottles of dry-mixed 98140 maize powders, analysed by rt-PCR using a sample intake of 150 mg

CRM	Number of samples analysed	Relative between bottle heterogeneity ($s_{bb, rel}$) [%]	Relative maximum hidden heterogeneity ($u^*_{bb, rel}$) [%]
ERM-BF427b	N = 15, n = 5	4.5	2.6
ERM-BF427c	N = 18, n = 5	1.4	1.4
ERM-BF427d	N = 18, n = 5	1.3	1.0

The relative maximum heterogeneity of the materials was therefore 4.5 %, 1.4 % and 1.3 % for ERM-BF427b, ERM-BF427c and ERM-BF427d, respectively. These results were used to calculate the standard uncertainty contribution of the materials' homogeneity to the total uncertainty budget (Table 6).

4.2 Minimum sample intake

A mass of 100, 150 and 200 mg powder was employed throughout this certification project for DNA extraction by the CTAB method. The assumption that this amount of substance is representative for the whole material was investigated by using 150 mg of the maize seed powders for homogeneity assessment, and therefore it is advised to use sample intakes not smaller than 150 mg.

5 Stability

5.1 Short-term stability

In order to assess whether special care must be taken during transportation, a short-term stability of dried maize seed powder was investigated using an isochronous approach [14]. ERM-BF427d was chosen for this study as it contained the highest GM mass fraction of the 98140 maize powders (nominal 100 g/kg). Five bottles were stored at each of the temperatures 18 °C and 60 °C during 2 and 4 weeks, and three subsamples from each bottle were analysed (N = 5, n = 3). The same number of reference samples was likewise stored at -70 °C. Genomic DNA was extracted from the samples by the CTAB method, the extractable DNA content was determined by the PicoGreen® method and visualised by gel electrophoresis. No DNA degradation was seen in any of the samples. Each DNA extract was analysed in triplicate by event-specific rt-PCR to reveal changes in GM quantification and the data were corrected for the different DNA content in the GM and non-GM powders (Figure 4). Scrutinising the data obtained, no outliers (95 % confidence level) were detected by Grubbs tests. Regression analysis was performed for each of the storage temperatures to reveal any trend in GM quantity in relation to the time of storage. A visual trend was observed after storage at 18 °C for 4 weeks, but it was not significant according to a *t*-test (at 95 % confidence level), and no trend was detected after storage at 60 °C for a time period of 4 weeks. This statement can reasonably be extended to ERM-BF427a, ERM-BF427b and ERM-BF427c given the similar composition of the four CRMs.
To conclude, the ERM-BF427 materials can be shipped under ambient conditions.

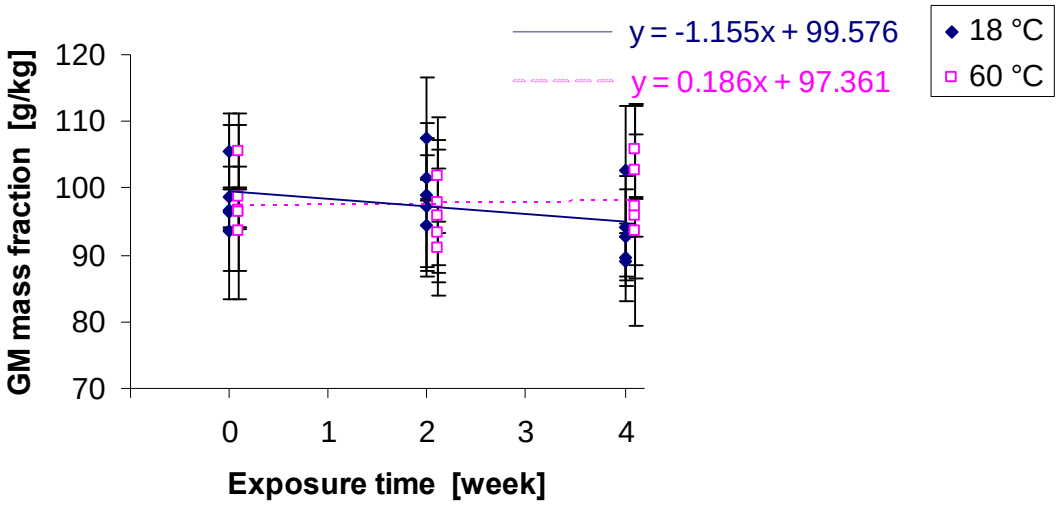

Figure 4: Short-term stability of ERM-BF427d stored at 18 °C (solid line) and 60 °C (dashed line) for 2 and 4 weeks and analysed by event-specific rt-PCR. The exposure time 0 week refers to the results obtained for samples stored at the -70 °C reference temperature during the 4-weeks study. The bars indicate the interval $\bar{x} \pm s$ for N = 5; n = 3. The measurements were calibrated with GM seed powder and data were corrected for the difference between the DNA mass extracted from the GM and non-GM seed powders.

5.2 Long-term stability

The stability of the maize seed powder was unaffected by short-term incubation at elevated temperatures (Section 5.1), similarly to what was observed for other maize matrices in the past. There is also no reason to think that the CRM would behave differently than other maize seed CRMs processed the same way during long-term storage at 4 °C under controlled conditions. Therefore, it was decided to rely on IRMM's stability monitoring experience. The long-term stability of maize CRMs during storage has been monitored at IRMM for more than 7 years, using ELISA and/or event-specific rt-PCR methods (Figure 5, based on unpublished results). The statistical analysis revealed that there was no statistically significant trend (95 % confidence level). The relative standard uncertainty of the long-term stability (u_{lts}) [14], calculated from the available maize stability data, was approximately 1.1 % of the certified value for a shelf life of one year and was used as the contribution due to instability of the CRMs upon storage in the total uncertainty budget (Table 6, Section 6.3).

In conclusion, the results demonstrate that the storage conditions of maize seed powder CRMs at IRMM are suited for long-term storage. In addition, post-certification monitoring is being carried out at regular intervals in order to check the stability of ERM-BF427. The stability results can reasonably be extended to other GM mass fractions of maize powder.

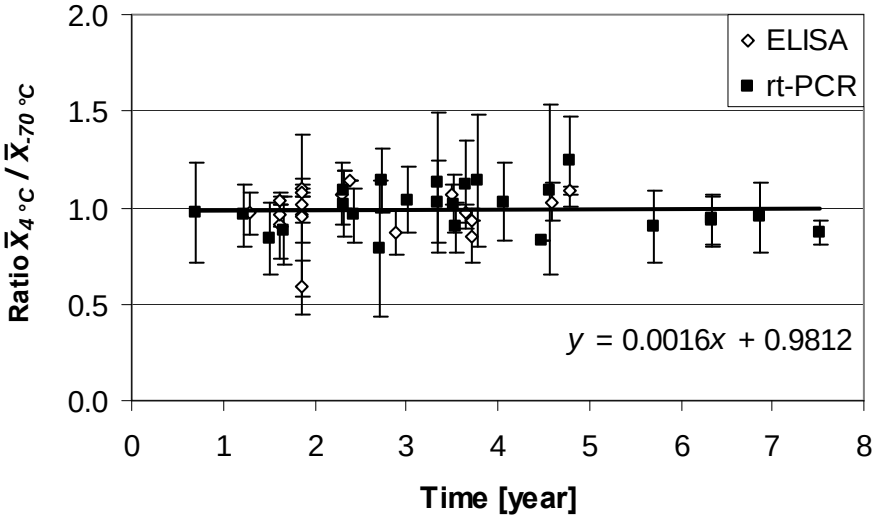

Figure 5: Long-term stability of different dried maize seed powder (not 98140) stored at 4 °C for various time periods, based on ELISA (o) and rt-PCR (■) measurements. The stability is expressed as the ratio between the GM mass fraction ratio in samples stored at 4 °C and that in samples stored for the same time period at the reference temperature (- 70 °C), with the bars indicating the expanded uncertainty interval ± *U* (*k* = 2). Each bullet corresponds to the average of 2 to 9 measurements. The bold line is the regression line generated on the basis of all data points. The monitoring was performed with the nominal 1 % GM maize powders of ERM-BF411 (Bt176), BF412 (Bt11), BF413 (MON 810), BF414 (GA21), BF415 (NK603), BF416 (MON 863), BF417 (MON 863xMON 810), BF418 (1507), BF420 (3272), BF423 (MIR604), BF424 (59122).

6 Certified mass fractions and uncertainty budgets

6.1 Certified value

The four CRMs under the label ERM-BF427 are maize seed powder CRMs processed from non-GM and GM base materials. While ERM-BF427a is prepared from the non-GM blank material, ERM-BF427b, c, d are gravimetrically produced mixtures of the non-GM and GM seed powders. The certified value is based on the GM mass fraction of dry-mixed GM and non-GM seed powder, corrected for their respective water mass fractions, and taking into account the powders' purity with regard to the 98140 event. If we assume that the purity of the non-GM powder is 100 %, which was supported by the data, the GM mass fractions can be calculated according to the following formula:

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

where

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

and

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

(p_{GM} = purity of the GM powder used for the dilution; WMF = Water Mass Fraction; anhyd = anhydrous)

For the purity of the GM base material, randomly selected seeds have been checked for the presence of the 98140 event (Section 3.1). No evidence of the occurrence of non-GM seeds among the GM seeds was found (data from Pioneer and IRMM). Based on a statistical analysis of the distribution of the probability to find a negative seed in the GM base material, it could be concluded that the purity was higher than 98 % (95 % confidence level). For the calculation of the certified value, a GM purity of the seed batch of 100 % was used, based on the actual number of positive seeds detected per total number of seeds analysed (200 out of 200 seeds tested). Similarly, the non-GM purity was taken as 100 % as no contamination was found during analysis of the corresponding seed batch.

6.2 Uncertainty budget

Controlled processing techniques in combination with purity controls of the GM and non-GM seeds and the derived base materials allow certifying the GM mass fractions in the CRMs with relatively low uncertainties (Table 6).

The combined standard uncertainty of the certified value comprises contributions from the between-bottle heterogeneity at the recommended sample intake of 150 mg (u^*_{bb}), the long-term stability of the material (u_{lts}, calculated for 12 months) and the characterisation of the materials (u_{char}). The u_{char} includes uncertainties related to the weighing procedure, the determination of the water mass fraction in the powders, and the analysis of the purity of non-GM and GM base materials (Table 6). To calculate the expanded uncertainty corresponding to a 95 % level of confidence a coverage factor of 2 was used [15].

For the blank material, the LOD of the method (and not the LOQ) was used to describe the 95 % confidence interval on the certified value (< 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 0.12 g/kg, which is below the LOD.

The uncertainty contribution of the long-term stability testing has been estimated by monitoring nominal 10 g/kg maize seed powder CRMs by ELISA or rtPCR over time (Figure 5). A coverage factor *k* of 2 was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % (Table 6).

Table 6: Uncertainty budget for the mass fraction of 98140 maize in ERM-BF427

ERM	Certified value [g/kg]	Standard uncertainty contributions [g/kg]						Expanded uncertainty U (k = 2) [g/kg]
		s_{bb} [2]	u_{lts} [3]	$u_{char,1}$ [4]	$u_{char,2}$ [5]	$u_{char,3}$ [6]	$u_{char,4}$ [7]	
BF427a	**< 0.4** [1]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
BF427b	**5.0**	0.2250	0.0561	0.0146	0.0084	0.1155	0.0216	0.6
BF427c	**20.0**	0.2800	0.2244	0.0540	0.0291	0.1155	0.0865	0.8
BF427d	**100**	1.3000	1.1217	0.2182	0.1188	0.1155	0.4323	4

[1] With a 95 % probability, the certified value is below this level. The LOD was used to calculate the certified property of the blank material according to the GUM [15].
[2] Standard uncertainty contribution resulting from the heterogeneity assessment, derived from Table 5.
[3] Standard uncertainty resulting from the stability of dried maize seed powders during storage, 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, based on the standard uncertainty of the method (7 g/kg) and the highest water mass fraction found in any of the powders used for mixing (12 g/kg for the non-GM powder).
[6] Standard uncertainty introduced by the purity 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 introduced by the purity of the GM base material (> 98 %), based on the half-width of the interval between 98.5 % and 100 % divided by the square root of 3 (rectangular distribution).

7 Intended use and instructions for use

ERM-BF427a, b, c, d are intended for use as quality control materials or calibrants in DNA-based methods for the measurement of GM material in food and feed. However, one has to be careful to draw quantitative conclusions in DNA copy number, for instance, from measurements of unknown samples as DNA-based GM quantification may vary with the particular matrix and maize variety tested [10].

The recommended minimum sample intake is 150 mg.

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

8 Metrological traceability

ERM-BF427a, ERM-BF427b, ERM-BF427c and ERM-BF427d are four reference materials certified for the mass fraction of 98140 maize seed powder. The certified mass fractions are based on gravimetric dry-mixing of non-GM maize seed powder with 98140 maize seed powder. The certified value is traceable to the 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.

The user of the certified reference material should, however, bear in mind that the values for the DNA copy number ratio measured by rt-PCR could potentially differ from the certified GM mass fraction as a result of different DNA mass extracted from a given sample intake GM and non-GM seed powders. Depending on the variety composition of the unknown sample measured in connection with ERM-BF427, rt-PCR measurement results of ERM-BF427 and the unknown sample may differ (average ± *U*) up to (33 ± 4) %. This difference may arise from the DNA mass difference between the sample intake of non-GM and GM seed powders used for the production of ERM-BF427 and may depend also on the DNA extraction method selected. The user should bear in mind that DNA extraction differences may additionally arise from the unknown sample depending on the composition of this sample and that these two effects may be additive (Section 3.5).

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Abstract

This report describes the processing and certification of four maize seed powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) DP-∅9814∅-6 maize (ERM-BF427a, b, c, d). The materials were processed and certified in 2008 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), according to the principles of ISO Guide 34.

Heterozygous seeds of GM DP-∅9814∅-6 maize and of a comparable non-GM maize variety were washed, dried and ground to GM and non-GM base powders. A non-GM material and three gravimetric mixtures of non-GM and GM maize seed powder (containing respectively 5, 20 and 100 g/kg GM maize) were prepared by dry-mixing. The material was then milled a second time. The certified values of these CRMs were calculated from the gravimetric preparations, taking into account the GM 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 four CRMs belonging to the ERM-BF427 set are certified to contain the following DP-∅9814∅-6 maize mass fractions:

CRM	**Certified value: 98140 maize mass fraction [1] [g/kg]**	**Uncertainty [2] [g/kg]**
ERM-BF427a	< 0.4 [3]	-
ERM-BF427b	5.0	0.6
ERM-BF427c	20.0	0.8
ERM-BF427d	100	4

[1] The certified value is based on the mass fraction of DP-∅9814∅-6 maize seed powder mixed in non-genetically modified maize seed powder and taking into account their respective DP-∅9814∅-6 maize purity and their water mass fraction. The certified value is traceable to the SI.
[2] 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 %.
[3] With a 95 % probability, the value of the material is below this level.

The CRMs are intended to be used for quality control or calibration of methods for the quantification of the DP-∅9814∅-6 maize mass fraction in food and feed. The CRMs are available in glass vials containing 1 g of dried maize powder closed under argon atmosphere. The minimum amount of sample to be used per analysis is 150 mg.

The four CRMs have been accepted as European Reference Material

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