Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

The certification of reference materials of maize seed powder with different mass fractions of the maize event 3272

Certified Reference Materials ERM®-BF420 (ERM®-BF420a, ERM®-BF420b, ERM®-BF420c)

Report EUR 22698 EN

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
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The certification of reference materials of maize seed powder with different mass fractions of the maize event 3272

Certified Reference Materials ERM®-BF420 (ERM®-BF420a, ERM®-BF420b, ERM®-BF420c)

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SUMMARY

This report describes the processing and certification of three maize powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) Event 3272 maize (ERM®-BF420a, ERM®-BF420b and ERM®-BF420c). The CRMs were processed and certified in 2006-2007 by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium.

Hybrid, hemizygous seeds of Event 3272 maize and of its near-isogenic non-GM counterpart were dried and ground to GM and non-GM base powders in a two-step grinding process. A non-GM pure material and two gravimetric mixtures of non-GM and GM maize powder were prepared by dry-mixing. 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.

The three CRMs belonging to the ERM-BF420 set were certified to contain the following Event 3272 maize mass fractions:

CRM	Certified value: Event 3272 maize mass fraction [1)] [g/kg]	Uncertainty [2)] [g/kg]
ERM-BF420a	< 1.3 [3)]	-
ERM-BF420b	9.8	1.2
ERM-BF420c	98	8

[1)] The certified value is based on the mass fraction of Event 3272 maize powder mixed in non-genetically modified maize powder and taking into account their respective Event 3272 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 [1] 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 Event 3272 maize mass fraction in food and feed. The CRMs are available in glass bottles containing 1 g of dried maize powder closed under argon atmosphere. The minimum amount of sample to be used per analysis is 100 mg.

The three CRMs (ERM-BF420a, ERM-BF420b and ERM-BF420c) have been accepted as European Reference Material® (ERM®) after peer evaluation by the partners of the European Reference Materials consortium [2].

TABLE OF CONTENTS

GLOSSARY

$\bar{x}$	average
Event 3272	GM maize event containing the genes for a thermostable α-amylase (Amy797e [1]) and for phosphomannose isomerase (Pmi [1])
ANOVA	analysis of variance
b	slope in the equation of linear regression $y = a + bx$
CRM	Certified Reference Material
CTAB	cetyltrimethylammonium bromide
Ct-value	number of PCR cycles to pass a set cycle threshold
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERM®	trademark of European Reference Materials
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
rt-PCR	real-time PCR
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 rt-PCR
T-DNA	transfer DNA, i.e. the transgenes-containing DNA fragment transferred to the plant during *Agrobacterium*-mediated genetic transformation
u	standard uncertainty
U	expanded uncertainty
u^*_{bb}	standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
u_{lts}	standard uncertainty contributed by the long-term stability of the material
Zm *adh1* [1]	*Zea mays* L. alcohol dehydrogenase *adh1* gene

[1] Following international nomenclature guidelines, three-letter non-italic codes with a capital letter at the beginning refer to the protein, whereas lowercase italic letters are used for the genes; a two-letter prefix referring to the plant species is non-italicised and separated from the gene name by a space.

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) that are authorised in accordance with Community legislation [3]. In general, this necessitates on the one hand the development and validation of reliable detection methods, and on the other hand the production of reference materials for the quality control and calibration of methods for the quantification of the GMO.

Syngenta Crop Protection AG (Basel, CH) has developed the genetically modified (GM) maize Event 3272. Following Commission Regulation (EC) No 65/2004 [4], the Event 3272 maize received the unique identifier SYN-E3272-5. This maize event has been genetically engineered to express two new proteins, a chimeric α-amylase enzyme from the archael order Thermococcales (Amy797e) and the phosphomannose isomerase (Pmi or ManA) protein used to provide a selection advantage during tissue culture. The Event 3272 maize is intended for mixing into other maize batches prior to processing by the dry-grind ethanol process, whereby the expressed Amy797e α-amylase enzyme will catalyse the hydrolysis of starch into smaller and less complex carbohydrate molecules. This should obviate the need for addition of purified α-amylases.

The Institute for Reference Materials and Measurements (IRMM, Geel, BE) was asked by Syngenta Crop Protection AG to develop and produce a reference material for the Event 3272 maize. The major objective of the project was, therefore, the production of certified reference materials (CRMs) containing different mass fractions of the genetically modified Event 3272 maize seed.

2 CRM processing

2.1 Characterisation of the base materials

For the preparation of the CRMs, Syngenta Seeds Inc. (Bloomington, IL, USA) supplied non-modified maize seeds and Event 3272 maize seeds to IRMM. The Event 3272 maize seeds are hybrid seeds produced by open-field pollination. The non-GM comparator line is a near-isogenic hybrid maize variety resulting from conventional breeding. Quality control was done on both seed lots by Syngenta, following the International Rules for Seed Testing 2006 [5] for testing the genetic and analytical purity, and according to AFNOR [6] for analysis of the adventitious presence of GMOs. Using total protein fingerprint analysis by isoelectric focusing, 100 % of GM seeds (n = 176) conformed with both parent seeds. Similarly, 175 out of 176 non-GM seeds (99.4 %) were hybrids of the breeding parents, with the remaining single seed being the result of a self-pollination. Furthermore, no seeds other than maize kernels (larger than one-half the original size) were identified in 1 kg of both seed batches. With regard to the general adventitious presence of GM events, including Event 3272, no GM material was detected by rt-PCR in 1 kg (corresponding to approximately 3000 seeds) of non-GM seeds tested (lower limit of quantification 1 g/kg). For the GM maize lot, no adventitious presence of GM events, other than Event 3272, was reported when analysing 1 kg of seeds. Furthermore, 293 out of 299 random GM seedlings tested positive for the synthetic α-amylase gene (*amy797E*) by rt-PCR; because all of these contained less than 1.5 copies of the *amy797E* gene relative to the reference gene, it was concluded that they are heterozygous for the transgene. The remaining 6 seedlings tested negative for the same gene and were therefore non-transgenic. This reflects a GM purity of the GM maize seed lot of at least 96 % (Poisson distribution for rare events; 95 % confidence level).

The reported purity and genetic composition of both maize seed batches were verified at IRMM by analysing 50 randomly selected GM seeds and 50 randomly selected non-GM seeds for the presence of the GM Event 3272. In order to avoid influences from attached dust particles on the analytical results, seedlings were grown and genomic DNA was extracted from the leaves using the DNeasy Plant Mini Kit (QIAGEN, Hilden, DE). Quantitative rt-PCR was performed using primer pairs and labelled TaqMan® probes specific for Event 3272 or for the maize reference gene encoding alcohol dehydrogenase (Zm *adh1*) [7]. Detection was done on an ABI7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [8]. The results confirmed that all 50 plants from the GM batch contained Event 3272. Similarly, all plants from the non-GM batch had a GM mass fraction below the limit of detection (LOD = 7.4 g/kg, mean of 2 experiments). Statistical analysis (Poisson distribution for rare events) revealed that the non-GM and the GM maize seed batch both had a genetic purity > 94 % (95 % confidence level) with regard to the absence and presence of Event 3272 respectively, confirming the companies' results.

After arrival, the maize seeds were stored at 4 °C in the dark until use. Twenty five kg of non-GM maize seeds and ten kg of Event 3272 maize seeds were used for the processing of ERM-BF420.

2.2 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 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 bags flushed with argon.

The maize kernels were rinsed in demineralised water, drained, and dried under vacuum at 30 °C during approximately 23 h. This resulted in a water mass fraction loss of 25 to 30 g/kg (measured by volumetric KFT). 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, resulting in a water mass fraction of 9 g/kg and 14 g/kg for the non-GM and GM material respectively (n = 3; measured by volumetric KFT). The powder was ground a second time under the same conditions, followed by another drying step under vacuum at 30 °C. For the second grinding step a sieve insert was used with 0.5 mm mesh width. Slow feeding of the mill ensured that the whole base material passed the sieve, thus excluding selection during grinding. The temperature of the mill was constantly monitored and milling was stopped for a while before the temperature exceeded 40 °C. Each finally ground base material was mixed in a turbula mixer for 30 minutes to improve equal distribution of the different parts of the maize tissue separated by the milling process. The final water mass fraction was 12.9 and 12.2 g/kg (n = 3) for the non-GM and GM powder respectively, and they were kept in closed plastic containers.

2.3 Gravimetric preparation of GM mixtures

The ground base materials were used to produce two powder mixtures containing mass fractions of Event 3272 maize powder at nominal levels of 10 and 100 g/kg. The powder materials were weighed using a calibrated balance with a relative standard uncertainty lower than 0.1 %. The masses of non-GM and GM maize powders required to produce these mixtures were corrected for the water mass fraction of the starting materials. First, the mixture for the nominal mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM ground base materials. The starting materials were combined in one container, turbula-mixed for 30 min, and further mixed in a special dry-mixing device for another 2 min. The 10 g/kg mass fraction was produced in the same way by further dilution of the 100 g/kg GM powder with pure non-GM powder.

2.4 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. Rubber stoppers were automatically placed in the bottle neck. Before 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 GM levels: nominal 0 g/kg = silver, nominal 10 g/kg = red, nominal 100 g/kg = brown, consistent with the cap colours of previous IRMM CRMs. Following inventorying, the bottles were brought to a storage room for long-term storage in the dark at 4 °C.

2.5 Processing control

The residual mass fraction of water was determined by volumetric KFT in randomly selected bottles from each of the powder mixtures (Table 1). As a result of the drying steps during the processing, the water mass fractions in the final CRM powders were relatively low (below 20 g/kg). For all three CRMs, a trend was, however, observed in the water mass fractions as a function of the bottling order, starting from about 15.9 g/kg residual water in the bottles filled during the first day, 13.8 g/kg in those filled during the second day, and 8.5 g/kg in bottles filled during the third day. We hypothesised that this trend reflects a combination of water uptake during storage of the bottles after filling and before final closure under argon (which was only done after all bottles of the particular CRM were filled) and water loss during the final vacuum drying before closure of the bottles under argon. As the water mass fractions were low in all individual bottles analysed, no negative effect on the stability of the CRM bottles is expected. The water uptake of the maize powder was further

investigated by hygroscopy analysis on open CRM bottles (of ERM-BF420c), showing a 22 ± 2 g/kg ($\overline{x}$ ± s) mass fraction increase after exposure for 1 h to a relative humidity of 43 % (n = 2), and a 31 ± 2 g/kg ($\overline{x}$ ± s) increase at a relative humidity of 75 % (n = 2). The customer is, therefore, informed on the certificates that these CRMs may take up water after opening of the vials and that this may affect their stability.

Table 1: Water mass fraction in ERM-BF420 CRMs determined by volumetric KFT (*N* = 10, *n* = 1)

CRM	Water mass fraction [g/kg]	
	$\overline{x}$	s
ERM-BF420a	14.7	3.6
ERM-BF420b	11.8	3.4
ERM-BF420c	12.0	3.3

Five randomly selected bottles from each of the powder mixtures were analysed for their particle size distribution based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). From each bottle, 3 subsamples were analysed. The powders had very similar particle size distribution profiles, with an average median particle size of 120 ± 14 μm ($\overline{x}$ ± s; based on the data of all three CRMs). The vast majority of particles in all three CRMs was below 730 μm, and only 0.05 % had a larger size (up to 1030 μm) (this is based on the equivalent volume diameter fraction; see legend of Figure 1). The particle size distribution of ERM-BF420a is shown in Figure 1 as an example. The average particle size of 120 μm was used for the calculation of the minimum sample intake for the three CRMs (Section 3.2).

Maximum and average particle sizes were also confirmed by sieve analysis using ten sieves with meshes ranging from 32 μm to 710 μm. Each CRM was analysed once (N = 1), using the combined contents of 10 bottles to reach the recommended sample intake of 10 g. The maximum particle size was below 710 μm for all three CRMs and 52-54 % of the particles had a size below 180 μm. It is concluded from the results of both particle size analysis methods that the powders are sufficiently fine for an adequate extraction of genomic DNA [9].

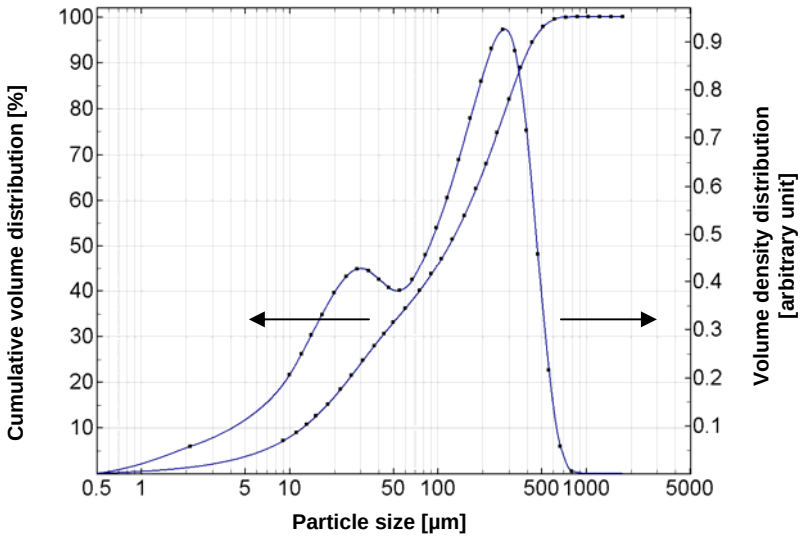

Figure 1: Average particle size distribution in ERM-BF420a (*N* = 5, *n* = 3).
The cumulative volume distribution of particles derived from laser light scattering data (left Y axis) is based on their equivalent volume diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. These data were converted into a volume density distribution curve (right Y axis) showing the distribution of particles according to particular size classes, expressed in an arbitrary unit.

ERM-BF420b and ERM-BF420c are mixtures of the pure GM and non-GM maize powders, produced gravimetrically and certified for their GM powder mass fraction. Quantification of the GM content is, however, based on rt-PCR, which measures DNA copy number ratios. To verify if the GM DNA copy number fraction remained conserved in the gravimetric powder mixtures, the mass fraction of DNA in both pure base materials was investigated. The DNA mass was determined by a slight modification of the classical fractionation method developed by Ogur & Rosen [10]. Following the sequential removal of ethanol-, ethanol-ether- and acid-soluble compounds, the DNA was obtained by repeated acidic extraction with 0.84 mol/L perchloric acid at 70 °C. The mass of DNA was measured spectrophotometrically after derivatisation with diphenylamine, which reacts specifically with 2-deoxyriboses linked to purine nucleobases [10, 11]. To reveal a difference between both powders, the ratio of their extractable DNA mass fractions was calculated as follows:

$$\frac{\text{Extractable mass of DNA in 100 mg Event 3272 maize seed powder}}{\text{Extractable mass of DNA in 100 mg non - GM maize seed powder}}$$

In the ground GM and non-GM materials, the respective DNA mass fraction was respectively 0.31 ± 0.01 and 0.29 ± 0.02 µg/mg ($\bar{x}$ ± s), resulting in a DNA mass fraction ratio of 1.05 (Table 2). A *t*-test confirmed that there was no significant difference in the DNA mass fraction between the powders (95 % confidence level). Although this may suggest that the certified GM powder mass fractions equal corresponding GM copy number fractions, the customer is reminded that IRMM currently only certifies these materials for their GM powder mass fraction.

Table 2: Total DNA mass fraction extracted from GM and non-GM maize powder

Extraction method	*n*	Ratio of DNA mass fraction in GM/non-GM	*U* (*k* = 2)
Modified Ogur & Rosen [10]	9	1.05	0.06

To verify the consistency of the certified mass fractions in these CRMs, the mass fraction of Event 3272 maize in all three CRMs was analysed by rt-PCR targeting the specific T-DNA insertion in this maize. Genomic DNA was extracted from 100 mg powder samples using a modified CTAB method [12], with the cesium chloride purification step replaced by an additional extraction with chloroform and a final ethanol precipitation. Real-time PCR measures copy numbers of the targeted DNA sequences but is calibrated with mass fractions of pure Event 3272 maize powder. Genomic DNA extracted from the pure GM powder is diluted in water and analysed by rt-PCR, producing a calibration curve ranging from 0.1 to 25 % mass fraction GM maize. The absolute GM mass fraction was furthermore related to the mass fraction of the maize-specific *adh1* reference gene, calculated from a calibration curve of the same pure Event 3272 maize DNA diluted in water (mass fraction interval 1 to 100 %). The GM quantity is therefore expressed as a relative mass fraction. The efficiency of the amplification was determined from the slope of the regression line between the calibrant's mass fractions and the obtained Ct-values; for all standard curves, the efficiency was within the limits of the rt-PCR control chart. The limit of detection (LOD) was calculated as $(3.3*s)/b$, with s representing the standard deviation for the results of a triplicate reaction on the lowest GM mass fraction analysed and b the slope of the calibration curve. The results of the GM quantification for the three CRMs are shown in Table 3. Quantification of the GM mass fraction in the three CRM powders by rt-PCR confirmed the consistency of the gravimetrically prepared mass fractions of ERM-BF420. However, one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements of unknown samples as DNA- and/or protein-based GM quantification may vary with the particular matrix and the maize seed variety tested [13].

Table 3: GM quantification in ERM-BF420 CRMs by event-specific real-time PCR

CRM	Event 3272 mass fraction [1)] [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF420a	< 1.3 [2)]	-
ERM-BF420b	10.4 [3)]	2.0
ERM-BF420c	98.1 [3)]	19.7

[1)] Real-time PCR measures copy numbers of the targeted GM DNA sequence in relation to copy numbers of the endogenous reference gene, calibrated with DNA extracted from pure GM powder and diluted in water.
[2)] The measured value was below the LOD of the method (1.3 g/kg) for all three subsamples from five bottles (N = 5, n = 3) of which each was measured in three replicates.
[3)] Average of the rt-PCR results obtained on the subsamples from a number of bottles (N = 15, n = 4 for BF420b; N = 20, n = 3 for BF420c), with each subsample measured in three replicates.

3 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 mixtures. The adequacy of the dry-mixing technology for the preparation of maize mixtures with different GM mass fractions has been shown before using maize materials processed in the same way as described for the Event 3272 maize [14]. Here we only report on the results of a homogeneity study performed on each of the two Event 3272 maize mixtures. Additionally, the recommended minimum sample intake is discussed.

3.1 Homogeneity study

The homogeneity of ERM-BF420 with respect to the Event 3272 maize mass fraction was investigated by rt-PCR using bottles selected according to a random stratified procedure. For ERM-BF420b, the homogeneity study consisted of fifteen CRM bottles which were analysed under repeatability conditions using a sample intake of 100 mg powder. For ERM-BF420c, data from the short-term stability study (Section 4.1) were used to assess homogeneity (comprising 5 reference samples and twice 5 samples stored during 2 and 4 weeks respectively at 4 °C); there was no trend observed for these samples in the stability study (Section 4.1).

Grubbs tests were performed to detect outlying individual results as well as bottle averages. No outliers were detected for any of the materials.

Regression analyses were 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.

It was furthermore checked whether the data followed a normal or unimodal distribution using normal probability plots and histograms respectively. The individual data and the bottle averages for both CRMs followed a normal distribution.

ANOVA statistics were used to calculate the between bottle standard deviation (s_{bb}) and the maximum uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), using the formulas [15]:

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

(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 larger of both values was included into the overall uncertainty of the certified values (Section 6.2).

Table 4: Calculation of uncertainties due to heterogeneity between bottles of dry-mixed Event 3272 maize CRMs, analysed by rt-PCR using a sample intake of 100 mg

CRM	Number of samples analysed [1)]	Relative between bottle heterogeneity ($s_{bb, rel}$) [%]	Relative maximum hidden heterogeneity ($u^*_{bb, rel}$) [%]
ERM-BF420b	N = 15, n = 8	3.9	2.2
ERM-BF420c	N = 15, n = 3	1.0	3.6

[1)] From each of 15 bottles (N), a number of subsamples (n) were analysed by rt-PCR using three replicates.

3.2 Minimum sample intake for analysis

Many commonly employed DNA extraction methods for plant powders recommend the use of 100 mg of powder as sample intake. The assumption that this quantity ensures a sufficient homogeneity was investigated.

The mass density of the non-GM maize seed powder was determined by so-called tap-density measurements using the procedure described in [16]. Taking into account the mass density (0.80 g/mL) and the particle size distribution (average particle size of 120 µm), it was calculated that a 100 mg sample roughly contains 1.4×10^5 powder particles. Consequently, 100 mg of ERM-BF420b (nominal 10 g/kg) would still contain 1400 GM particles.

As a general rule, it is advised to use sample intakes not smaller than 100 mg.

4 Stability

4.1 Short-term stability

In a previous study, the short-term stability of dried maize powder (ERM®-BF416 certified for its MON 863 maize GM mass fraction) was investigated by isochronous incubation of bottles at 60 °C for 2 and 8 weeks and by analysis of DNA integrity and GM mass fraction in the samples [14]. It was concluded that dried maize powder CRMs could be shipped under ambient conditions without affecting their stability.

Because the Event 3272 maize used to produce the ERM-BF420 CRMs expresses a thermostable α-amylase which could potentially start degrading the starch in the powders during incubation at elevated temperatures, we investigated the short-term stability of this CRM using an isochronous approach [17]. ERM-BF420c was chosen for this study as it contains the highest GM mass fraction of the Event 3272 maize CRMs (nominal 100 g/kg). Five bottles were incubated at either 4 °C, 18 °C or 60 °C during 2 and 4 weeks, and three subsamples from each bottle were analysed (N = 5, n = 3). A similar number of reference samples was likewise incubated at -70 °C. Genomic DNA was extracted from the samples by the modified CTAB method (described in the paragraph below Table 2) and visualised by gel electrophoresis. No substantial DNA degradation was seen in any of the samples. Each DNA extract was also analysed in triplicate by event-specific rt-PCR to reveal changes in GM quantification (Figure 2). Scrutinising the data obtained, three outliers (95 % confidence level) were detected by Grubbs tests (two in the 2 weeks/4 °C data set and one in the 4 weeks/18 °C set), but these were retained as no technical reason to exclude them was found. Regression analysis was done for each of the incubation temperatures to reveal any trend in GM quantity in relation to the time of incubation. A t-test showed that none of the data sets indicated a significant change over the time period of 4 weeks (95 % confidence level).

It was concluded that the uncertainty of degradation during dispatch is negligible for all three CRMs. ERM-BF420 can be shipped under ambient conditions.

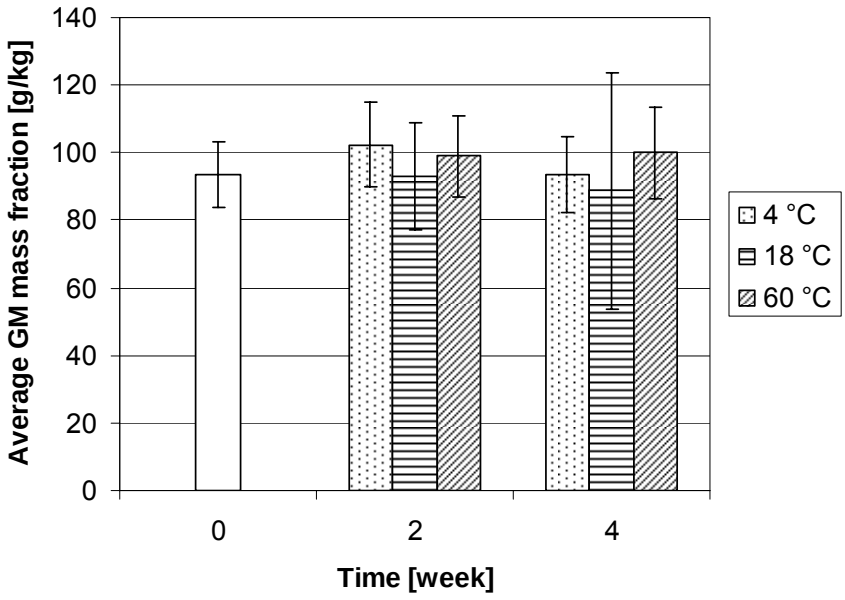

Figure 2: Short term-stability of ERM-BF420c stored at different temperatures for 0, 2 and 4 weeks and analysed by event-specific rt-PCR. The column shown for 0 weeks refers to the results obtained for samples stored at the -70 °C reference temperature during the 4-weeks study. The bars indicate the interval $\bar{x} \pm s$ for N = 5; n = 3.

4.2 Long-term stability

The stability of the Event 3272 maize powder was unaffected by short-term incubation at elevated temperatures (Section 4.1), similarly to what was observed for other maize matrices in the past [14]. There is also no reason to think that the Event 3272 maize CRM would behave differently than other maize CRMs during long-term storage under controlled conditions. Therefore, it was decided to rely on IRMM's stability monitoring experience with maize powder CRMs. The long-term stability of maize CRMs (ERM-BF411 till BF418) during storage has been monitored at IRMM for a total of more than 6 years, using ELISA and/or event-specific rt-PCR methods (Figure 3, based on own unpublished results). There was no significant trend in the stability data over the time period investigated (*t*-test, 95 % confidence level). The relative standard uncertainty of the long-term stability (u_{lts}) [18], calculated from the available maize stability data, was approximately 1.7 % of the certified value per year.

Post-certification monitoring is being carried out at regular intervals in order to further check the stability of ERM-BF420.

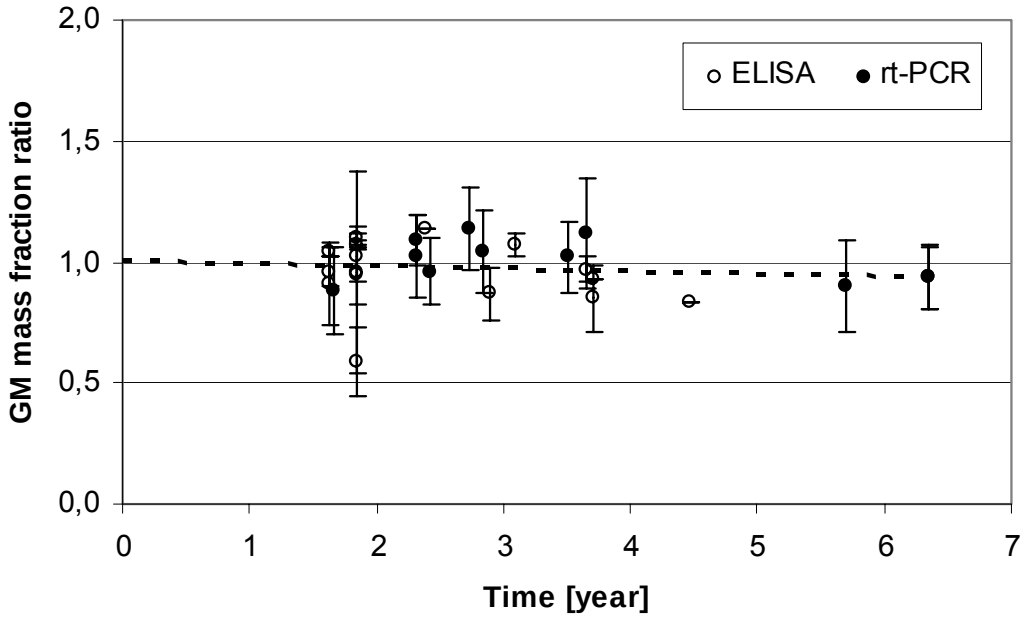

Figure 3: Long term-stability of different maize CRMs (ERM-BF411 till BF418) stored at 4 °C for various time periods, based on ELISA and rt-PCR measurements. The ratio is shown between the GM mass fraction obtained for samples stored at the reference temperature (- 70 °C) and that for samples stored at 4 °C, with the bars indicating the expanded uncertainty interval ± *U* (*k* = 2). The dotted line is the regression line generated on the basis of all data points.

5 Characterisation

The three CRMs under the label ERM-BF420 are maize powder CRMs processed from pure non-GM and pure GM base materials. While ERM-BF420a is prepared from the pure non-GM blank material, ERM-BF420b and ERM-BF420c are mixtures of the pure non-GM and GM powders that were produced gravimetrically. The certified value is based on the mass fractions of dry-mixed powders, corrected for their water mass fractions and GM purity, and was later verified by rt-PCR (Table 3).

The seed batches used for the processing of these powders were thoroughly checked for any genetic impurity. No indication of the presence of the Event 3272 was found in the non-GM seed lot by rt-PCR, confirming the quality control results of the company supplying the seeds (Section 2.1). Processing controls additionally confirmed that the powder used for the production of ERM-BF420a did not contain traces of the Event 3272 above the LOD of the applied rt-PCR method (Table 3).

For the GM seeds, no indication was found for the absence of the Event 3272 in any of the individual seedlings raised from the GM seed lot when measured by event-specific rt-PCR; quality control on a larger number of seedlings by the company supplying the seeds revealed only a low percentage of seedlings that carried no transgene (Section 2.1). Processing controls confirmed that ERM-BF420b and ERM-BF420c contained the expected copy number fraction of the Event 3272 (Table 3).

6 Certified mass fractions and uncertainty budgets

6.1 Certified value

The certified value is 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 are corrected for their respective water mass fractions and their estimated Event 3272 maize purity. The GM mass fractions (Table 5) are calculated as:

$$\frac{\text{corrected mass GM powder}}{(\text{corrected mass GM powder}) + (\text{corrected mass non-GM powder})} * 1000 \text{ g/kg}$$

For the purity of the GM base material the genetic identity of randomly selected kernels has been checked (Section 2.1). A low incidence of the occurrence of non-GM seeds among the GM seeds was found (data from Syngenta). 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 96 % (95 % confidence level). For the calculation of the certified value (Table 5), the observed GM purity of 98 % was used, based on the actual number of positive seeds detected per total number of seeds analysed (293 out of 299 seeds tested).

The purity of the non-GM base material was checked by analysis of the genetic identity of randomly selected kernels (Section 2.1). No evidence could be found that seeds of the GM Event 3272 were among the non-GM seeds. The certified value for ERM-BF420a is therefore based on the LOD of the rt-PCR method applied (1.3 g/kg). However, as no evidence for a contamination was found, a 0 % non-GM powder purity was used for the calculation of the certified GM mass fraction of ERM-BF420b and c.

Table 5: Certified mass fractions of Event 3272 maize in ERM-BF420 CRMs

CRM	Certified value [g/kg]
ERM-BF420a	**< 1.3** [1)]
ERM-BF420b	**9.8**
ERM-BF420c	**98**

[1)] With a 95 % probability, the true value of the material is below this level.

6.2 Uncertainty budget

Controlled processing techniques in combination with purity controls of the GM and non-GM seeds and the derived powder base materials allow certifying the GM mass fractions in the CRMs with rather low uncertainties.

The combined standard uncertainty of the certified value comprises contributions from the weighing procedure, the water mass fraction in the powders, the between-bottle inhomogeneity at the recommended sample intake of 100 mg, the long-term stability of the material (calculated for 12 months) and 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 [19].

Table 6: Uncertainty budget for the mass fraction of Event 3272 maize in ERM-BF420

CRM	Standard uncertainty contribution [g/kg]						Expanded uncertainty *U* (*k* = 2) [g/kg]
	u_1 [1)]	u_2 [2)]	u_3 [3)]	u_4 [4)]	u_5 [5)]	u_6 [6)]	
ERM-BF420a	n.a.	n.a.	n.a.	n.a.	0.3637	n.a.	**-**
ERM-BF420b	0.0189	0.0004	0.3798	0.1660	0.3637	0.0792	**1.2**
ERM-BF420c	0.1334	0.0029	3.4988	1.6596	0.3637	0.7922	**8**

[1)] Standard uncertainty of the mass determination, mainly based on the uncertainty of the balance and the number of weighing steps required.
[2)] Standard uncertainty of the water mass fraction determination by volumetric KFT, based on the standard uncertainty of the method (1.41 g/kg) and the highest water mass fraction found in any of the powders used for mixing (14.7 g/kg for the blank material).
[3)] Standard uncertainty contribution resulting from the homogeneity assessment (Table 4).
[4)] Standard uncertainty resulting from the stability of dried maize seed powders during storage, extrapolated to 12 months.
[5)] Standard uncertainty introduced by the purity of the non-GM base material (LOD = 1.3 g/kg), based on the half-width of the interval between 0 and 1.3 g/kg, divided by the square root of 3 (rectangular distribution).
[6)] Standard uncertainty introduced by the purity of the GM base material (> 96 %), based on the half-width of the interval between 96 % and 100 % divided by the square root of 6 (triangular distribution).

7 Metrological traceability

ERM-BF420a, ERM-BF420b and ERM-BF420c are three reference materials certified for the mass fraction of Event 3272 maize powder. The certified values are based on gravimetric dry-mixing of non-modified maize seed powder with Event 3272 maize seed powder.

The respective certified values are traceable to the SI. The traceability chain to the kilogram is based on the use of calibrated balances, a thorough control of the weighing procedure and the control of the purity of the used seeds.

The user of the certified reference material should, however, bear in mind that the GM copy number fraction measured by rt-PCR could potentially deviate from the certified GM mass fraction as a result of different DNA extraction efficiencies from GM and non-GM powders.

8 Intended use and instructions for use

ERM-BF420a, b and c are intended for use as quality control materials or calibrants in DNA- or protein-based methods for the detection of genetically modified material in food and feed.

The recommended minimum sample intake is 100 mg.

The powders are hygroscopic. Bottles should be stored dry and in the dark at maximum 4 °C.

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The certification of reference materials of maize seed powder with different mass fractions of the maize event 3272, ERM®-BF420 (ERM®-BF420a, ERM®-BF420b, ERM®-BF420c)
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Abstract

This report describes the processing and certification of three maize powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) Event 3272 maize (ERM®-BF420a, ERM®-BF420b and ERM®-BF420c). The CRMs were processed and certified in 2006-2007 by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium.

Hybrid, hemizygous seeds of Event 3272 maize and of its near-isogenic non-GM counterpart were dried and ground to GM and non-GM base powders in a two-step grinding process. A non-GM pure material and two gravimetric mixtures of non-GM and GM maize powder were prepared by dry-mixing. 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.

The three CRMs belonging to the ERM-BF420 set were certified to contain the following Event 3272 maize mass fractions:

CRM	**Certified value: Event 3272 maize mass fraction [1] [g/kg]**	**Uncertainty [2] [g/kg]**
ERM-BF420a	< 1.3 [3]	-
ERM-BF420b	9.8	1.2
ERM-BF420c	98	8

[1] The certified value is based on the mass fraction of Event 3272 maize powder mixed in non-genetically modified maize powder and taking into account their respective Event 3272 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 [1] 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 Event 3272 maize mass fraction in food and feed. The CRMs are available in glass bottles containing 1 g of dried maize powder closed under argon atmosphere. The minimum amount of sample to be used per analysis is 100 mg.

The three CRMs (ERM-BF420a, ERM-BF420b and ERM-BF420c) have been accepted as European Reference Material® (ERM®) after peer evaluation by the partners of the European Reference Materials consortium [2].

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