Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of Reference Materials of Maize Seed Powder containing Genetically Modified MON 810 Maize

Certified Reference Materials ERM®-BF413k (ERM®-BF413ak, ERM®-BF413ck, ERM®-BF413ek, ERM®-BF413gk)

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GLOSSARY

ANOVA	analysis of variance
b	slope in the equation of linear regression $y = a + bx$
conf.	confidence
CRL-GMFF	Community Reference Laboratory for Genetically Modified Food and Feed
CRM	Certified Reference Material
cp	number of copies
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
hmg	maize-specific reference gene, i.e. the single-copy maize gene encoding the high mobility group I protein
LOD	limit of detection
LOQ	limit of quantification
MON 810	GM maize (*Zea mays* L.) event MON 810 containing a 3'-truncated c*ryIAb* gene from *Bacillus thuringiensis*, providing resistance to the European corn borer, under the control of the enhanced CaMV 35S promoter and *hsp*70 leader sequences; terminator sequence absent
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
R^2	correlation coefficient
RSD	relative standard deviation
s	standard deviation
s_{bb}	standard deviation between bottles
SI	International System of Units
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation during real-time PCR
U	expanded uncertainty
u_{bb}	standard uncertainty related to the between-bottle heterogeneity
u^*_{bb}	standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
u_{char}	standard uncertainty related to the characterisation
u_{lts}	standard uncertainty related to the long-term stability of the material
$\bar{x}$	average

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 GMO quantification methods, and on the other hand the production of reference materials for the quality control and calibration of these methods.

GMO Certified Reference Materials (CRMs) from the Institute for Reference Materials and Measurements (IRMM, Geel, BE) have been produced by mixing genetically modified (GM) powder with non-GM powder. Until recently, these matrix CRMs were exclusively certified for their GM mass fraction. In October 2004, the European Commission issued a recommendation to express the content of GM food and feed as the percentage of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes [2]. The GM DNA copy number ratio, expressed in %, is determined according to the following formula:

$$\text{GM DNA copy number ratio [\%]} = \frac{\text{GM DNA copy numbers [cp]}}{\text{Target taxon-specific DNA copy numbers [cp]}} \times 100$$

Consequently, the IRMM is certifying the existing and new GMO CRMs for their GM DNA copy number ratio in addition to their GM mass fraction.

Monsanto Company (St Louis, MO, USA) has developed the genetically modified (GM) maize line MON 810, providing resistance against the European corn borer. Following Commission Regulation (EC) No 65/2004 [3], the MON 810 maize received the unique identifier code MON-ØØ81Ø-6. MON 810 maize has been produced through microparticle bombardment of tissues using plasmid DNA. Detailed investigations on the selected MON 810 transgenic line revealed the presence of a single insert, composed of a 3'-truncated c*ryIAb* gene from *Bacillus thuringiensis* under the control of the enhanced CaMV 35S promoter and hsp70 leader sequences. No terminator sequence was present as a result of the 3' truncation resulting from the transformation process.

In 2001, the IRMM was asked by Monsanto Company to develop and produce a reference material for the quantification of MON 810 maize. The resulting CRM was named ERM-BF413 [4]. As the original CRM batch is exhausted, IRMM decided to produce a new series of MON 810 maize reference material. The major objective of the project was, therefore, the production of certified reference materials (CRMs) containing different mass fractions of the genetically modified MON 810 maize seed. This new series received the code ERM-BF413k with the affix 'k' referring to the replacement batch. There are, however, a few important differences between the two CRM series:

1. The seeds used for the production of ERM-BF413k originated from a different GM and a different conventional maize variety.

2. The MON 810 event in the hybrid GM seeds used was derived from the male parent in the case of ERM-BF413k, while it originated from the female parent in ERM-BF413.

3. The maize powders used for ERM-BF413 were cryo-ground using liquid Nitrogen, while the maize powders for ERM-BF413k were ground using Nitrogen gas to pass the material through the mill. ERM-BF413 was therefore composed of a finer powder.

4. The new CRM series of ERM-BF413k contains different nominal GM mass fractions compared to the earlier ERM-BF413 series.

5. In case of ERM-BF413k the CRM ERM-BF413ek (certified to contain 19.8 g/kg GM mass fraction) was certified for its GM DNA copy number ratio; in case of ERM-BF413 the CRM ERM-BF413d (certified to contain 10 g/kg GM mass fraction) was certified for its GM DNA copy number ratio [5].

The user is, therefore, reminded that ERM-BF413k and ERM-BF413 can not be compared when expressing real-time PCR measurement results in mass fractions.

2 Participants

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- Neotron S.p.A., Modena, Italy (SINAL, 26)*
- Crop Research Institute, Prague, Czech Republic (Czech accreditation institute, 8/2007)*
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* Measurements within the scope of accreditation to ISO/IEC 17025.

3 CRM processing

3.1 Characterisation of the base materials

For the preparation of the CRMs, Monsanto Company supplied conventional maize seeds of variety EXP258B and MON 810 maize seeds of variety DKC57-84 to IRMM. The GM variety was produced by crossing a male MON 810 line with a female non-GM line. It should be reminded that for the previous MON 810 CRM (ERM-BF413), the GM event was introduced into the hybrid seeds from the female side. Quality control on the GM seed lot was done by Monsanto by qualitative PCR for MON 810 on 352 individual seeds. The results (**Table 1**) showed that the lot purity was above 97.4 % (95 % confidence level). Furthermore, similar qualitative tests indicated with 95 % confidence that the GM seed lot impurity was below 0.99 % for GA21 and MON 863 and below 0.3 % for NK603. The lot impurity of the conventional maize was below 0.99 % for MON 810, GA21, MON 863 and NK603 (95 % confidence level).

After arrival, the maize seeds were stored at 4 °C in the dark until use. Forty kg of non-GM maize seeds and ten kg of MON 810 maize seeds were used for the processing of ERM-BF413k.

The reported purity and genetic composition of both maize seed batches were verified at IRMM by analysing 52 randomly selected GM seeds and 51 randomly selected non-GM seeds for the presence of the GM event MON 810. In order to avoid influences from attached dust particles on the analytical results, seedlings were grown and genomic DNA was extracted from the leaves. Quantitative real-time PCR was performed according to the event-specific real-time PCR method [6], using genomic DNA from pure MON 810 maize powder for calibration. Detection was done on an ABI7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [7]. The results, summarised in **Table 1**, showed that one out of 52 plants from the GM batch did not contain the MON 810 event. All plants from the non-GM batch had no signal above the limit of detection (0.9 g/kg). Statistical analysis (Poisson distribution for rare events) revealed that the non-GM and the GM maize seed batch had a genetic purity > 94.1 % and > 90.7 % (95 % confidence level), respectively.

Real-time PCR measurement on the non-GM maize powder revealed that no MON 810 traces above the detection limit of the method applied (0.9 g/kg) are present (**Table 4**).

The seed lot purity data and the real-time PCR data were taken into account for the calculation of the uncertainties on the certified values of the reference materials produced (Section 7.3).

Table 1: Genetic purity of the non-GM and GM seed batches used for the processing of ERM-BF413k with respect to GM event MON 810

Batch	Test results reported by	Number of seeds	Number of GM positives	Number of GM negatives	Lot purity (95 % conf.)
Non-GMO	Monsanto	300[1)]	0	300[1)]	> 99.0 %
	IRMM	51	0	51	> 94.1 %
GMO	Monsanto	352	348	4	> 97.4 %
	IRMM	52	51	1	> 90.7 %

[1)] Four pools of 75 seeds were tested by qualitative PCR

3.2 Production of the base materials

The GM and non-GM base materials were processed separately and essentially followed the processing approach used for ERM-BF413 [4]. Cross-contamination and contamination with foreign DNA were avoided using glove box systems and clean laboratory clothing. All contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to exposure to the base materials. An in-house validation study had proven beforehand that the solution degraded DNA effectively under the given conditions. If required, the base powders were stored for short time periods in closed plastic containers.

The maize seeds were rinsed in water, drained, and dried under vacuum at 30 °C. The non-GM seeds were processed in two batches and the GM seeds in one batch. Drying resulted in a water mass fraction loss of 11-29 g/kg for the different seed batches. The dried seeds were then milled using a high impact mill with a triangular ribbed open grinding track in order to obtain the ground base material. The high impact mill was flushed with nitrogen gas throughout the milling process and milling was interrupted if the temperature rose above 40 °C. An additional vacuum drying at 30 °C was carried out to further reduce the water content of the once ground base material. This resulted in a water mass fraction of 14.2 ± 1.7 and 9.6 ± 3.3 g/kg for batch 1 and 2 of the non-GM material (n = 2) and 11.5 ± 1.8 g/kg for the GM material (n = 3), measured by volumetric KFT. The powders were ground a second time under the same conditions, followed by a second drying step under vacuum at 30 °C. For the second grinding step a sieve insert with a mesh size of 0.5 mm was used. Slow feeding of the mill ensured that the whole base material passed the sieve, thus excluding selection during grinding. Each ground base material was mixed in a Dynamic CM-200 mixer (WAB, Basel, CH) for 30 minutes to improve equal distribution of the different parts of the maize tissues separated by the milling process. At this stage, both batches of milled non-GM powder were mixed in order to obtain one batch of non-GM powder material. The water mass fraction in the final base seed powders was 36.6 ± 2.3 g/kg and 23.0 ± 0.5 g/kg for the non-GM and GM powder, respectively (n = 3), an increase resulting from water uptake during mixing. The powders were stored in closed plastic containers until further use.

3.3 Gravimetric preparation of GM mixtures

The ground base materials were used to produce a GMO blank material and three mixtures containing different mass fractions of MON 810 maize seed powder in non-GM maize seed powder at nominal levels of 5, 20 and 100 g/kg. All four materials, including the blank powder, were treated according to the same procedure. The powder materials were weighed using a calibrated balance with a relative standard uncertainty lower than 0.1 %. The starting materials were combined in one container, mixed in the Dynamic CM-200 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 mass of the non-GM and GM powder was corrected for its respective water mass fraction. The nominal GM mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM ground base materials. The 20 g/kg GM mass fraction was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder, and the 5 g/kg mixture was likewise produced by further diluting the 20 g/kg mixture with non-GM powder; at each mixing step, the water mass fraction of the mixed materials was taken into account. The gravimetric preparation formed the basis for the calculation of the mass fraction of the powders (see Section 6.1).

3.4 Bottling

The powders were bottled in 10 mL brown glass vials using an automatic filling device. The first 30 bottles of each batch were discarded as an additional precaution against carry-over contamination. Lyophilisation inserts were automatically placed in the bottle neck. Before final closure of the vials, air was evacuated in a freeze-drier and replaced by argon. The vials were closed inside the freeze-drier with the help of a hydraulic device and then sealed with aluminium caps to prevent accidental opening during storage and transport. Colour-coded caps were used for easy identification of the different GM levels: nominal 0 g/kg = silver (BF413ak), nominal 5 g/kg = blue (BF413ck), nominal 20 g/kg = green (BF413ek), nominal 100 g/kg = brown (BF413gk), consistent with the cap colours of previous IRMM CRMs. Each of the vials was identified by a numbered label indicating the ERM code (**Figure 1**). Following the inventorying and the selection of vials for future analysis according to a random stratified sampling scheme, the bottles were brought to a storage room for long-term storage in the dark at 4 °C.

Figure 1: Prototype labels for the ERM-BF413k series. The denotation "blank" was used for the nominal 0 g/kg MON 810 maize powder (BF413ak), while BF413ck, BF413ek and BF413gk refer to the nominal 5 g/kg, 20 g/kg and 100 g/kg MON 810 maize, respectively.

3.5 Processing control

The residual mass fraction of water in randomly selected bottles from each of the powder mixtures was determined by volumetric KFT (**Table 2**). No significant difference was found between the mean water mass fractions in the different CRMs. 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 ERM-BF413k CRMs determined by volumetric KFT (*N* = 10, *n* = 1)

CRM	Water mass fraction [g/kg]	
	$\bar{x}$	*s*
ERM-BF413ak	21.2	2.7
ERM-BF413ck	22.7	4.6
ERM-BF413ek	22.4	2.9
ERM-BF413gk	19.5	5.1

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). It is important to understand that the cumulative size distribution of particles derived from laser light scattering data, as displayed in Figure 2, is based on their equivalent spherical diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. Since most particles are presumably not perfectly spherical, the volume-based presentation of the PSA data is, therefore, overestimating the average particle size. From each bottle, 3 subsamples were analysed. The powders had very similar particle volume distribution profiles (**Figure 2**), with a median particle size around 150 µm and a maximum particle size below 610 µm. The average particle volume, calculated by the PSA software, was 175 µm, 166 µm, 175 µm and 157 µm for ERM-BF413ak, ck, ek and gk, respectively.

It was concluded from the results of particle volume analysis that the powders are sufficiently fine for an adequate extraction of genomic DNA [5].

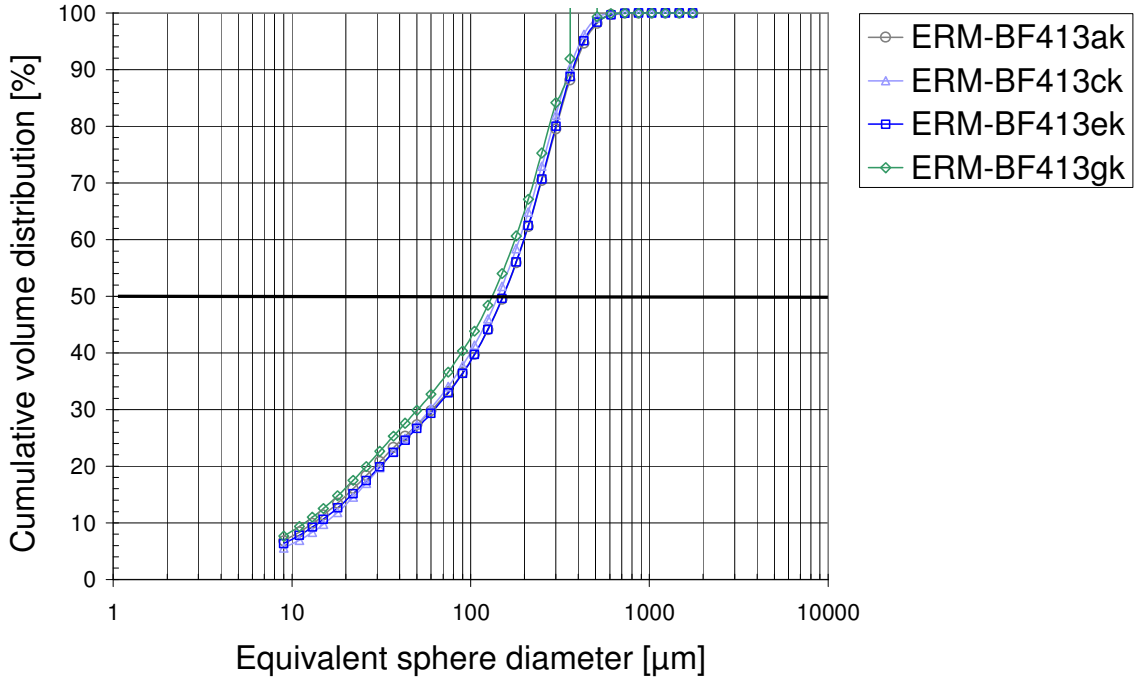

Figure 2: Average particle volume distribution in ERM-BF413k analysed by PSA (*N* = 5, *n* = 3).

Three of the described CRMs are mixtures of GM and non-GM maize powders, produced gravimetrically and used for quantitative measurements on the level of the genomic DNA, following DNA extraction. In order to provide an indication that the GM DNA copy number ratio (Section 7.2) was conserved in the gravimetric powder mixtures, the DNA mass fraction in both pure base materials was investigated. The DNA mass was determined by a slight modification of the classical fractionation method developed by Ogur and Rosen [8]. 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 estimated spectrophotometer after derivatisation with diphenylamine, which reacts specifically with 2-deoxyriboses linked to purine nucleobases [8, 9]. To reveal a difference between the two powders, the ratio of their extractable DNA mass fractions was calculated as follows, resulting in a ratio of 0.97 (**Table 3**):

$$\frac{\text{Extractable mass of DNA in 100 mg MON 810 maize powder}}{\text{Extractable mass of DNA in 100 mg non - GM maize powder}}$$

Table 3: Mass ratio of extracted DNA from GM and non-GM maize powders

DNA extraction method	*N*	Average ratio GM/non-GM	*U* (*k* = 2)
Modified Ogur & Rosen [8]	9	0.97	0.07

A *t*-test confirmed that there was no significant difference between the DNA mass fraction in the powders (95 % confidence level). Although this may, in theory, suggest that the certified GM powder mass fractions are equal to the corresponding GM DNA copy number fractions, the customer is reminded that IRMM only certifies these materials for their GM powder mass fraction, except for ERM-BF413ek. The latter CRM is additionally certified for its GM DNA copy number ratio based on an interlaboratory comparison (see Section 6.2).

As a further control for the gravimetric preparations, the mass fraction of MON 810 maize in all four CRMs was verified by real-time PCR targeting the transgenic DNA insertion in this maize [6]. Genomic DNA was extracted from 200 mg powder samples using the GeneSpin genomic DNA extraction method (Eurofins GeneScan GmbH, Freiburg, DE). The real-time PCR test was calibrated with genomic DNA extracted from the pure MON 810 maize powder, diluted in water to produce a calibration curve ranging from 4-times diluted to 2000-times diluted and from undiluted to 200-times diluted for the GM gene and reference gene, respectively. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' GM mass fractions and the obtained Ct-values; for all standard curves, the efficiency (at least 86 %) was within the limits of the real-time PCR control chart. The limit of detection (LOD) was calculated using the calibration curve approach [10]. The results of the GM quantification for the four CRMs are shown in **Table 4**. Although the calculated data deviated from the certified mass fractions, quantification of the GM mass fraction ratio in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF413k. However, 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 GM quantification may vary with the particular matrix and the maize variety tested [11].

Table 4: GM quantification in ERM-BF413k CRMs by event-specific real-time PCR

CRM	MON 810 maize mass fraction [g/kg] [1)]	U (k = 2) [g/kg]
ERM-BF413ak	< 0.9 [2)]	-
ERM-BF413ck	5.7	1.1
ERM-BF413ek	15.5	3.1
ERM-BF413gk	76.9	12.0

[1)] Average for five subsamples from each of five random bottles (N = 5, n = 5), with each subsample measured in three replicates. Genomic DNA from MON 810 seed powder was used for calibration.
[2)] The obtained value was below the LOD determined during method validation (0.9 g/kg).

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 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 MON 810 maize [12]. Here we report on the results of a homogeneity study performed on each of the three GM maize mixtures. Additionally, the recommended minimum sample intake is discussed.

4.1 Homogeneity study

The homogeneity of the MON 810 maize powder in ERM-BF413k with respect to the GM mass fraction and GM DNA copy number ratio was measured by real-time PCR, using the plasmid DNA calibrant ERM-AD413 for calibration and the event-specific MON 810 PCR detection method [6]. The homogeneity was investigated under repeatability conditions on bottles randomly taken from the entire batch. The number of bottles to be analysed was determined by the cube root of the total number of bottles produced per CRM, i.e. 15 (for BF413ck) or 19 bottles (for BF413ek and BF413gk). From each bottle five extracts were made and each analysed in triplicate. The DNA extraction method used was the GeneSpin method (GeneScan, GmbH, Freiburg, Germany) using a sample intake of 200 mg. The analyses were performed at the National Institute of Biology (NIB) in Slovenia.

The GM DNA copy number ratio determined in this study (mean ± *s*) was (0.19 ± 0.05) %, (0.72 ± 0.12) % and 3.55 ± 0.32 % for ERM-BF413ck, ek and gk, respectively. These DNA copy numbers can be converted into 37 ± 2 % of the numerical value of the GM mass fraction certified, which is in line with the expectations for hybrid maize seed produced by crossing a male homozygous GM variety with a female non-GM variety [13]. These values are only indicative values, as homogeneity studies are not meant for the determination of the true value of an analyte.

Two individual measurement data were missing from the results. One sample extraction did not yield any DNA, and during another extraction the GeneSpin column leaked; while DNA was obtained in the latter case, the real-time PCR result was considered unreliable and therefore excluded from the analysis. Among the remaining data, Grubbs tests were performed to detect outlying individual results as well as bottle averages. No outliers were detected among the sample means for any of the materials. For both ERM-BF413ck and ek, two outlying analytical data were found at the 95 %, but not at the 99 % confidence level. The outliers were not removed from the data sets as no technical reason for exclusion was found.

Regression analyses were used to evaluate potential drifts in the results. No significant trends were observed in the sample means according to the bottle number. Significant trends were, however, observed in the analytical sequence at the 95 % and 99 % confidence level for ERM-BF413ck and ERM-BF413ek and at the 95 % confidence level for ERM-BF413gk.

It was furthermore checked whether the data followed a normal or at least 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 bottle standard deviation (s_{bb}) and the maximum standard uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), using the formulas [14]:

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

(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 5**). The larger of both values was included into the calculation of the overall uncertainty on the certified values (Section 7.3).

Table 5: Standard uncertainties due to heterogeneity between bottles of dry-mixed MON 810 maize CRMs analysed by real-time PCR

CRM	Relative between bottle heterogeneity ($s_{bb,rel}$) [%]	Relative maximum hidden heterogeneity ($u^*_{bb,\,rel}$) [%]
ERM-BF413ck	7.7	4.2
ERM-BF413ek	- [1)]	3.2
ERM-BF413gk	2.0	1.6

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

4.2 Minimum sample intake for analysis

Many commonly employed DNA extraction methods for plant powders recommend the use of 100 or 200 mg of powder as sample intake.

Within the frame of the GM DNA copy number characterisation study on ERM-BF413ek (Section 6.2), it was shown that similar results were obtained using DNA extracted with either the CTAB extraction method using a sample intake of 100 mg powder or the GeneSpin method using a 200 mg sample intake. Therefore, the use of a minimum sample intake of 100 mg is suggested.

5 Stability

5.1 Short-term stability

In several previous studies on maize CRMs, the short-term stability of the powders was investigated by analysis of DNA integrity and GM DNA copy number ratio following isochronous incubation [16] of bottles at 4, 18 and 60 °C for several weeks (see certification reports of ERM-BF413d, BF416, BF420 and BF427, available at http://www.irmm.jrc.be). It was concluded that dried maize powder CRMs could be shipped under ambient conditions without affecting their stability.

The short-term stability of the new ERM-BF413k was similarly investigated following isochronous incubation of bottles at 18 and 60 °C for 2 and 4 weeks ($N = 5$). ERM-BF413gk was chosen for this study as it contains the highest GM mass fraction of the four maize CRMs (nominal 100 g/kg). From each of 5 bottles per condition, 5 subsamples were analysed for stability of the DNA in the matrix. A similar number of reference samples was likewise analysed following their incubation at a reference temperature of -70 °C during the 4 weeks. Genomic DNA was extracted from the samples by the GeneSpin method and visualised by gel electrophoresis. No substantial DNA degradation was seen in any of the samples. Each DNA extract was also analysed by event-specific real-time PCR [6] to reveal changes in GM quantification. The calibrant used in these experiments was ERM-AD413 (plasmid DNA) [17]. An average GM DNA copy number ratio of 4.03 ± 0.32 % was obtained for the reference samples (-70 °C) and a value of 3.98 ± 0.36 % was obtained if the data from all samples were considered (**Figure 3**).

Scrutinising the data obtained, the single and double Grubbs tests revealed no outliers (95 % confidence level). Regression analysis revealed no trend over the time period of 4 weeks for the samples incubated at 18 °C (*t*-test, 95 % confidence level). From the statistical analysis a trend towards higher values in relation to the incubation time was observed for the storage at 60 °C. The trend was significant at the 95 %, but not at the 99 % confidence level (slope of 0.053 for the GM DNA copy number ratio). Based on the existing experience about the stability of maize seed powders and considering that the differences were small, it was concluded that the observed trend at 60 °C did not reflect DNA degradation. Moreover, on the basis of the ratio between the mean result at 60 °C and that at 18 °C, no significant trend was observed in relation to the exposure time.

Therefore, it was concluded from the results obtained in this and in the previous studies that the uncertainty due to degradation during dispatch is negligible for all four CRMs. ERM-BF413k can be shipped under ambient conditions.

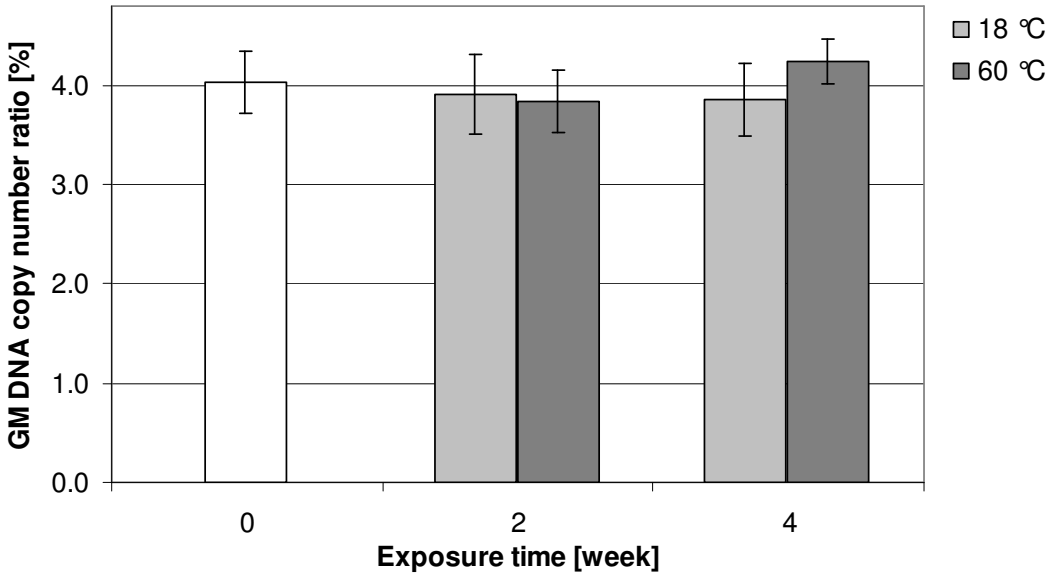

Figure 3: Short-term stability of ERM-BF413gk stored at 18 °C and 60 °C for 2 or 4 weeks and analysed by event-specific real-time PCR. The column shown for 0 weeks refers to the results obtained on samples stored at the -70 °C reference temperature during the 4-weeks study. The bars indicate the interval $\overline{x} \pm s$ ($N = 5$, $n = 5$).

5.2 Long-term stability

From post-certification stability analysis, the long-term stability of various maize CRMs, certified for their GM mass fraction, has been monitored at IRMM for a total of 7.5 years, using ELISA and/or event-specific real-time PCR methods (**Figure 4**, based on unpublished results). The uncertainty contribution from the long-term stability is estimated by calculating the uncertainty on the mass fraction ratio of samples stored at 4 °C and -70 °C ($\overline{x}_{4\,°C}/\overline{x}_{-70\,°C}$). Statistical evaluation revealed that there is no significant trend in the stability data (*t*-test, 95 % confidence level). The relative standard uncertainty of the long-term stability (u_{lts}) [18], calculated from the available stability data for all maize CRMs, was approximately 1.1 % of the certified value calculated for one year. It can be concluded that the storage conditions at IRMM are suited for the long-term storage of maize CRMs. As for all IRMM's CRMs, a post-certification monitoring scheme is put in place in order to continue monitoring the stability of ERM-BF413k.Q

For ERM-BF413, the stability monitoring has been evaluated from 2001 until 2005 by ELISA and only more recently by real-time PCR. Because the certified GM DNA copy number ratio for ERM-BF413ek is traceable to the MON 810 real-time PCR detection method [6], only the real-time PCR data from the stability monitoring of ERM-BF413 were used to calculate the standard uncertainty contribution from the stability to this certified value. Single and double Grubbs tests did not detect any outliers at a 95 % confidence level. The slope of the linear regression line was not significantly different from zero at a 95 % confidence level ($p = 0.32$). Accepting a shelf life of 12 months, a relative standard uncertainty contribution (u_{lts}) of 0.6 % for the stability was calculated. It is recommended to store the bottles at 4 ºC.

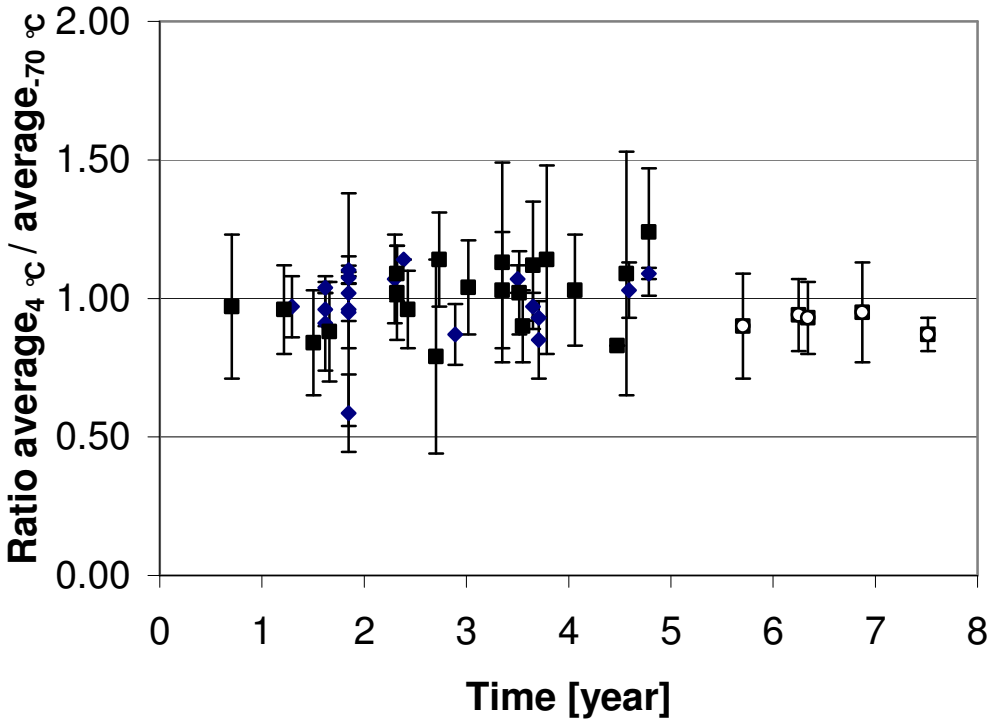

Figure 4: Long-term stability of dried maize seed powder CRMs stored at 4 °C for various time periods, based on ELISA (◆) and real-time PCR (■) measurements (*n* = 2 to 9). The real-time PCR data on ERM-BF413d (nominal 10 g/kg) are indicated with an open circle (○) within the black rectangle for real-time PCR. The stability is expressed as the ratio between the GM mass fraction 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).

6 Characterisation

The four CRMs under the label ERM-BF413k are maize powder CRMs processed from non-GM and GM hybrid seeds. While ERM-BF413ak is prepared from the pure non-GM blank material, the other CRMs of the ERM-BF413k series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. Two different certified parameters are envisaged during the characterisation study, GM mass fraction and GM DNA copy number ratio, and the analysis related to each of these is described below.

6.1 Characterisation related to GM mass fraction

The mass values are based on the GM mass fractions of dry-mixed GM and non-GM powder, corrected for their water mass fractions, and taking into account the powder's purity with regard to the MON 810 event. The values were calculated according to the following formulas:

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

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

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

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

For the purity of the GM base material the genetic identity of randomly selected seeds has been checked (Section 3.1). A minor occurrence of non-GM seeds among the GM seeds was found (data from Monsanto and IRMM, **Table 1**). 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 97 % (95 % confidence level). For the calculation of the certified value, a GM purity of the seed batch of 98.86 % was used, based on the actual number of positive seeds detected per total number of seeds analysed (348 out of 352 seeds tested).

In **Table 6**, the data supporting the calculation of the GM mass fractions are summarised.

Table 6: Stepwise dilution non-GM powder with GM MON 810 maize powder to process ERM-BF413k mixtures

ERM	GM powder			Non-GM powder[1)]	Mixtures
	Mass fraction [g/kg]	Water mass fraction [g/kg]	Mass [g]	Mass [g]	Resulting GM mass fraction [g/kg]
BF413gk[2)]	988.6	23.0	395.0	3605.0	98.9
	988.6	23.0	394.8	3605.0	98.9
BF413ek[2)]	98.9 [3)]	28.9	794.6	3205.0	19.8
	98.9 [3)]	28.9	795.0	3205.0	19.8
BF413ck	19.8 [3)]	25.1	991.2	3008.8	4.9
BF413ak	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 36.6 g/kg and was considered to be free of MON 810 maize.
[2)] Due to container size limitations, these preparations were weighed and mixed in two batches, the two batches were afterwards merged.
[3)] For the preparation of BF413ek and BF413ck, the 99 g/kg and 19.8 g/kg GM maize was used, respectively.

6.2 Characterisation related to GM DNA copy number ratio

In addition to the mass-based certification, one of the CRMs, ERM-BF413ek, is also certified for its GM DNA copy number ratio.

For the characterisation of ERM-BF413ek with respect to the GM DNA copy number ratio an interlaboratory study was organised. Sixteen laboratories were selected on the basis of price, proven experience and quality assurance systems in place. Each of the laboratories was asked to analyse one or two sample sets whereby each sample set consisted of two bottles of candidate ERM-BF413ek to be independently analysed on different days. The results obtained upon analysis of one sample set, therefore, comprise one data set (see below).

As an extensive previous study on a similar MON 810 reference material, ERM-BF413d, had shown that there was no difference between the results generated by three different DNA extraction methods [5], the participants of the current study had the choice between one of these three methods for DNA extraction. Eight participants (11 data sets) chose a CTAB based method according to ISO 21571:2005, Annex A3 [19] using a sample intake of 100 mg, 6 laboratories (9 data sets) used the silica-column-based GeneSpin method (GeneScan GmbH, Freiburg, Germany) using 200 mg sample intake, and one laboratory (2 data sets) used the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) requiring 5 DNA extractions of 20 mg per final extract to achieve a 100 mg minimal sample intake. Additionally, one laboratory used a fourth method, which was an alternative silica-column based DNA extraction method, the Roche High Pure GMO Sample Preparation kit (F. Hoffmann-La Roche Ltd, Basel, Switzerland), using 200 mg of powder (2 data sets). A total of 24 sample sets, i.e. 48 bottles of candidate ERM-BF413ek, were dispatched to the participants. In addition to the seed powders, the laboratories received one vial of ERM-AD413 (plasmid DNA) for calibration. All other reagents were provided by the participants.

Each of the two samples from one sample set were independently analysed on different days starting from DNA extraction and passing through to real-time PCR. Per sample, three DNA extracts were prepared ($N = 2$, $n = 3$); these were called the unknowns (U1 to U3 from sample 1 on day 1, U4 to U6 from sample 2 on day 2). The setup of the study was, therefore, as follows:

1 SAMPLE SET (→ 1 DATA SET)

= 2 samples (CRM bottles), analysed on 2 days

= 6 unknowns (DNA extracts)

= 18 PCR samples (3 DNA concentrations per unknown)

The DNA concentration was estimated by UV spectrometry, fluorometry (Picogreen or Hoechst H33258) or quantitative PCR according to the laboratories' preferred method. The DNA concentration of each DNA extract was adjusted to 20 ng/µL with nuclease-free water (undiluted unknowns) and the extracts were further diluted 2 x and 4 x to assess the possible occurrence of PCR inhibition. Undiluted and diluted unknowns were analysed in triplicate by event-specific real-time PCR. On each day, a fresh dilution series of the plasmid DNA calibrant ERM-AD413, ranging from 1 x 10^5 cp/µL to 5 cp/µL, was prepared using low TE buffer (0.1 mM Tris-HCl, 0.01 mM EDTA pH 8.0) [17]. The calibration standards used to compose the standard curve contained 500 000, 100 000, 10 000, 5000 and 1 000 copies per PCR reaction for the reference gene, and 50 000, 10 000, 1000, 100 and 25 copies for the GM gene. Real-time PCR experiments were carried out according to the manufacturer's instructions with all runs performed for at least 45 cycles.

Fifteen of the 16 laboratories returned data. Exclusion of data sets was based on the technical exclusion criteria described by Charels *et al.* [20], but modified and extended by the criteria listed below. The criteria used by Charels *et al.* included the PCR efficiency estimated on the basis of the slope of the calibration curve, the square of the correlation coefficient of the calibration curve ($R^2 \geq 0.98$), PCR inhibition analyses, confirmed technical mistakes and anomalies related to the DNA dilutions that were made. In this study the following changes were incorporated:

- The limits for the PCR efficiency of the calibration curves were arbitrarily set at ≥80 % and ≤ 120 % instead of the study-specific limits used by Charels et al. (mean efficiency ± 3 times *s*); use of the latter limits, however, would not have changed the number of accepted data. Furthermore, data were excluded if the PCR efficiencies obtained for the reference and the GM gene deviated > 20 %; this was not the case in this study.
- PCR inhibition effects in the calibration curves were assessed by plotting the mean Ct values against the logarithm of the DNA concentrations tested and calculating the Ct difference between the linear regression line and the measured Ct value; if the Ct difference was > 0.50 for the highest DNA concentration, the calibration interval was restricted by removal of this standard and the PCR inhibition analysis was repeated on the remaining standards. Every valid calibration curve must contain at least four calibration standards.
- As three DNA concentrations had been tested for the unknowns (20, 10 and 5 ng/µL), PCR inhibition in the unknowns was evaluated by comparing the measured Ct values with the linear regression line, obtained by plotting the mean Ct values against the logarithm of the DNA concentration for the three DNA concentrations; if the Ct difference for the undiluted extract was > 0.50, inhibition was inferred and the data for the undiluted extract were excluded. No data were excluded on the basis of the slope or correlation coefficient of the regression line obtained.
- An acceptance limit for the intermediate precision of data was introduced, based on the relative standard deviation (RSD) between the data obtained for day 1 and those for day 2 and also between data sets if the laboratory measured 2 sample sets. This RSD limit was set at ≤ 25 %.
- Individual data for the unknowns at the calibration limits were excluded if one of the replicate values was below the "mean – *s*" Ct value obtained for the lowest DNA concentration of the calibrant tested (calculated from the triplicates) or above the

"mean + *s*" Ct value obtained for the highest DNA concentration of the calibrant tested.

- A mean GM DNA copy number ratio for an unknown was only calculated if data for at least two of the three DNA concentrations had been accepted, i.e. were within the calibration interval for the GM and reference gene. A mean GM DNA copy number ratio for a data set was calculated only if data for at least two of the three unknowns per day had been accepted, i.e. a data set mean was calculated from the mean values of minimally four unknowns.

In accordance with the above-described exclusion criteria, five data sets from four laboratories were excluded. One data set was removed because three of the calibration curves had an efficiency below the minimal acceptance criteria and one of them had a squared correlation coefficient (R^2) of 0.97 which is below the minimal performance requirements ($R^2 \geq 0.98$) defined by the method validation guidelines of the CRL-GMFF [21]. Most of the individual data from this lab were also outside the calibration interval. The laboratory reported that this could have been caused by the use of rather old primers and probe solutions. Two other data sets, i.e. those from the laboratory using the Wizard DNA extraction method, were excluded because the RSD between both data sets (29 %) exceeded the set RSD limit of 25 %; therefore, the quality performance of the laboratory was questioned. One other data set (Roche extraction method) was excluded because the RSD between the two samples (44 %), exceeded the 25 % acceptance limit. The other data set from the laboratory that had applied the Roche extraction method was also excluded due to the fact that many individual data were outside the calibration interval, leaving too few data to calculate average copy numbers. Finally, one data set was excluded which had a mean GM DNA copy number ratio (0.38 ± 0.05 %) that was only half the mean value determined on the basis of the other laboratories' results; the laboratory was contacted for an explanation and proposed to repeat their analysis on new samples. The result obtained in the second experiment was 0.53 ± 0.13 %. The RSD between all sample means (26 %) exceeded the RSD exclusion limit, and, therefore, the performance of the laboratory to produce reliable measurements was questioned. Seventeen data sets were finally accepted, eight using the CTAB method, and nine using the GeneSpin extraction method (**Table 7**). In general, fluorimetry estimated the DNA concentration of the samples in the interval 45-100 ng/µL (mean per laboratory), while the concentrations measured by UV varied between 47 and 400 ng/µL. Using quantitative PCR for estimating the DNA concentration, the values obtained were rather low (mean of 26 ng/µL), resulting in copy numbers for the reference gene above the calibration interval for all undiluted extracts. Twelve individual data from undiluted extracts (from two data sets) were, therefore, removed from this laboratory. No other data were excluded from any other data set.

From the 17 accepted data sets, GM DNA copy number ratios were determined for the undiluted and for the 2 and 4 times diluted sample extracts. There was no indication of PCR inhibition for neither the calibrant, nor the unknowns, and no data were excluded on the basis of this criterion. A *t*-test (95 % confidence level) confirmed that there was no significant difference between the undiluted and the 2 or 4 times diluted samples when pooling all data. For each unknown, the mean GM DNA copy number ratio was calculated from the accepted data from the undiluted and both diluted samples. The mean of the GM DNA copy number ratio of the 6 unknowns was calculated and this value constitutes the GM DNA copy number ratio of each data set (**Table 7**). The certified GM DNA copy number ratio for ERM-BF413ek was calculated as the unweighted mean of all accepted data set means and the standard uncertainty on the characterisation (u_{char}) was determined from the standard deviation of the data set means (Section 7.3).

Table 7: Average GM DNA copy number ratio and standard deviation per data set.

Data set	DNA extraction method	GM DNA copy number ratio [%]	*s* [%]
1	CTAB	0.71	0.09
2	CTAB	0.94	0.05
3	CTAB	0.84	0.07
4	CTAB	0.83	0.13
5	CTAB	0.86	0.11
6	CTAB	0.64	0.07
7	CTAB	0.62	0.10
8	CTAB	0.73	0.14
9	GeneSpin	0.87	0.13
10	GeneSpin	0.68	0.11
11	GeneSpin	0.74	0.09
12	GeneSpin	0.81	0.13
13	GeneSpin	0.53	0.02
14	GeneSpin	0.64	0.10
15	GeneSpin	0.86	0.17
16	GeneSpin	0.82	0.03
17	GeneSpin	0.91	0.08
Whole study		0.77	0.12

The 17 data sets were subjected to further analysis. Scrutinising the data, no outliers were detected by the Dixon test and Grubbs test (95 % confidence level). However, data set 13 was detected as an outlier by the Nalimov *t*-test at the 95 %, but not at the 99 % confidence level. From the graphical presentation of the data set means (**Figure 5**), it can be seen that the standard deviation on this data set was very low. However, the same laboratory also analysed another data set (number 14), using the same DNA extraction method, which revealed a much higher standard deviation. Therefore, it was concluded that the standard deviation was not a good estimate for the uncertainty calculation on data set 13, and that the real uncertainty was probably much higher and within the overall uncertainty of the material. As a consequence, data set 13 was not considered an outlier. Also the Cochran test found no outliers on the basis of outlying data set variances. As can already be seen in **Figure 5**, the variances of the data sets were not homogeneous according to the Bartlett's test. Both skewness and kurtosis tests, however, indicated that the data were normally distributed (95 % confidence level).

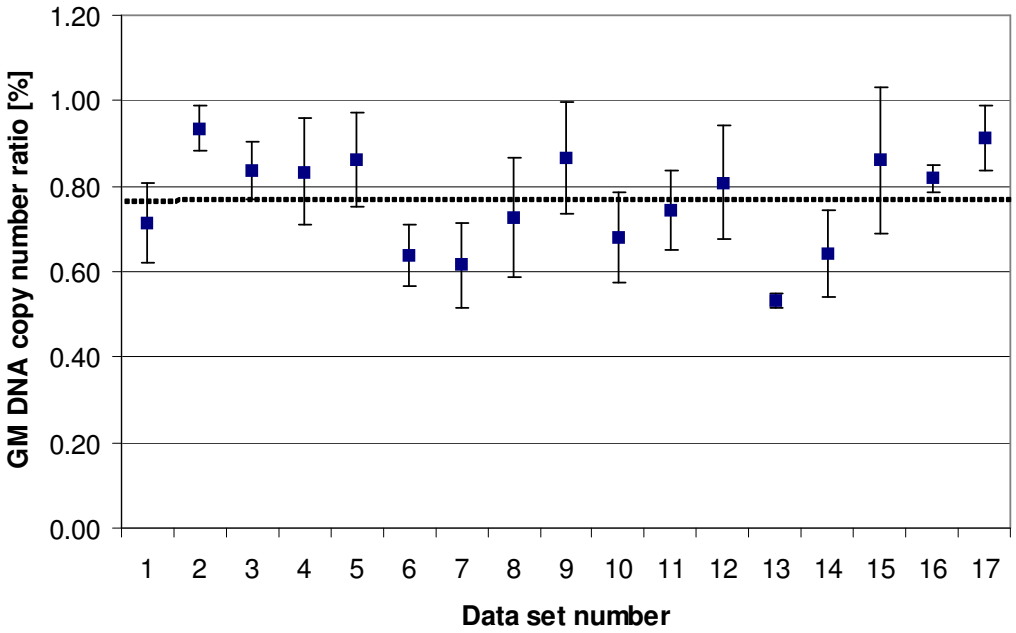

Figure 5: Data set means for the CTAB (1-8) and the GeneSpin (9-17) DNA extraction methods. Vertical bars denote 2*s*. The horizontal dashed line indicates the mean of the study.

PCR efficiencies and linearity of the calibration curves did not vary much between the data sets. A mean PCR efficiency of 98 ± 5 % was obtained and the R^2 value was 0.998 ± 0.003. There were no significant differences between the values obtained for the reference gene and for the GM gene, or between both DNA extraction methods or days of analysis (*t*-test, 95 % confidence level).

Figure 6 shows the mean GM DNA copy number ratio for each DNA extraction method and for each day of analysis. The DNA extraction method or the day of analysis did not impact the results of the study. A *t*-test confirmed that both variables had no significant effect on the GM DNA copy number ratio. However, ANOVA and a Snedecor *F*-test (99 % confidence level) showed significantly different GM DNA copy number ratios both between data sets and between samples. This variability illustrates the importance of selecting a sufficient number of independent data sets for the characterisation study.

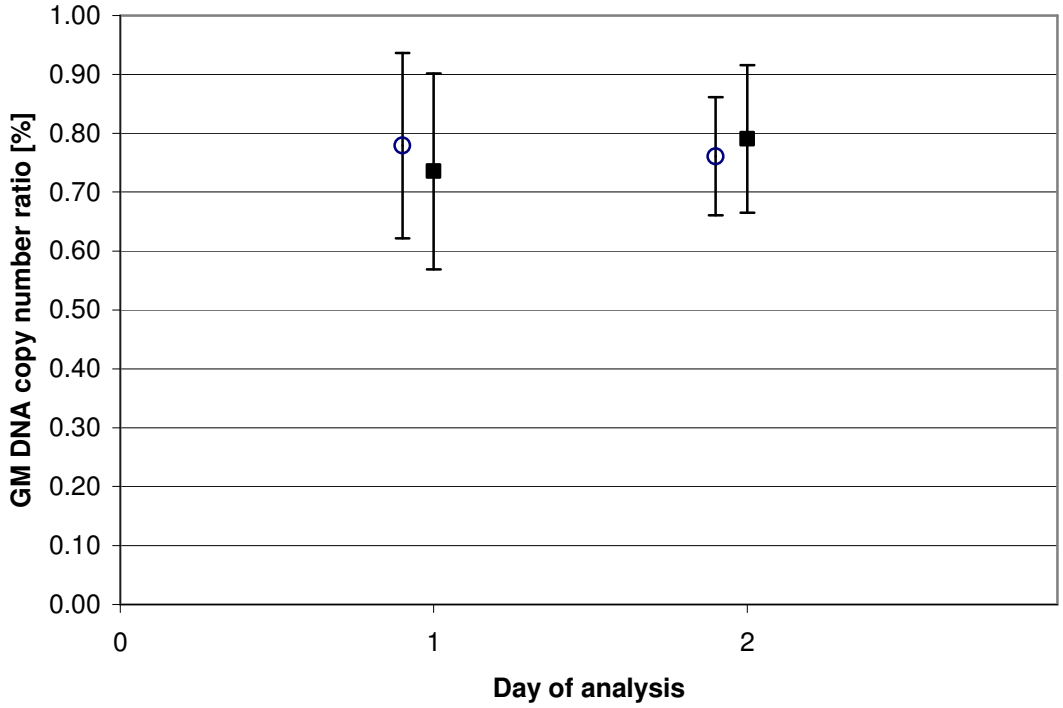

Figure 6: Mean GM DNA copy number ratio for each day of analysis for samples prepared by the CTAB (o) and the GeneSpin (■) DNA extraction method. Vertical bars denote 2*s*.

7 Certified values and uncertainty budgets

7.1 Certified value based on mass fraction

ERM-BF413k is a CRM certified for the mass fraction of MON 810 maize powder. 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 were corrected for their respective water mass fractions and their estimated MON 810 maize purity (**Table 6** in Section 6.1).

The seed batches used for the processing of these powders were thoroughly checked for any impurity. No indication of the presence of the MON 810 maize was found in the non-GM seed lot by real-time PCR (Section 3.1). Processing controls additionally confirmed that the powder used for the production of ERM-BF413ak did not contain traces of the MON 810 maize above the LOD of the applied real-time PCR method (**Table 4**). The certified value for ERM-BF413ak is based on the LOD of the real-time PCR method applied, as determined during in-house method validation. As no evidence for a contamination was found for the non-GM powder, a 100 % purity was used for the calculation of the certified GM mass fraction of the seed powder mixtures.

For the GM seeds, a low incidence of the occurrence of seed devoid of the MON 810 event was detected among the individual seedlings raised from the GM seed lot when measured by event-specific real-time PCR; quality control on a larger number of individual seeds by the company supplying the seeds confirmed this (Section 3.1). The determined purity of the GM seeds was taken into account when calculating the certified mass fraction according to the formula shown in Section 6.1. Real-time PCR measurements demonstrated that no mixing mistakes were done (**Table 4**).

7.2 Certified value based on GM DNA copy number ratio

The ERM-BF413ek material, characterised by a 19.8 g/kg GM mass fraction, was additionally certified for its GM DNA copy number ratio. This ratio is defined as the percentage of MON 810 DNA copy numbers in relation to maize-specific DNA copy numbers calculated in terms of haploid genomes [2] and calculated as follows:

$$\text{GM DNA copy number ratio [\%]} = \frac{\text{MON 810 DNA copy numbers [cp]}}{\text{maize-specific DNA copy numbers [cp]}} \times 100$$

Because the data are normally distributed (Section 6.2), the certified value is calculated as the unweighted mean of the data set means.

7.3 Uncertainty budget

The expanded uncertainty of the certified value (U_{CRM}) comprises standard uncertainty contributions from the characterisation, the inhomogeneity, and the stability [7].

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

The individual uncertainty contributions are summarised in **Table 8** and **Table 9**.

The uncertainty introduced by the inhomogeneity has been estimated as the relative standard deviation between units (s_{bb}) or the maximum heterogeneity potentially hidden by the method repeatability (u^*_{bb}) as defined in Section 4. The higher of these values was taken as a conservative estimate of heterogeneity. Note that the relative uncertainty due to heterogeneity is related to the certified quantity, which explains why this value is higher for ERM-BF413ek certified for GM mass fraction compared to the same material certified for GM DNA copy number ratio (**Table 8** and **9**, respectively).

The uncertainty contribution from the stability (u_{lts}) has been estimated on the basis of stability tests on maize CRMs and was calculated for 12 months (Section 5). The u_{lts} on the certified GM mass fraction was calculated from the long-term storage data of various maize CRMs, processed in the same way as ERM-BF413k. The u_{lts} on the certified GM DNA copy number ratio was calculated from the stability data obtained on ERM-BF413 only.

The u_{char} on the certified GM mass fraction 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 $u_{char,4}$).

For the certified GM DNA copy number ratio, u_{char} has been assessed by an interlaboratory comparison and was calculated as the standard error of the mean of data set means using the formula:

$$u_{char} = \frac{s}{\sqrt{d}}$$

where s = standard deviation of data set means

d = number of data sets

A coverage factor of 2 ($k = 2$) was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % [22].

For the certification based on GM mass fraction, it can be seen from **Table 8** that the standard uncertainty contribution introduced by the inhomogeneity is larger than the standard uncertainty contributions from characterisation and stability of the maize powders. For the certification based on DNA copy numbers (**Table 9**), the u_{char} has the largest contribution to the overall uncertainty.

For the blank material, the LOD of the method was used to describe the 95 % confidence interval on the certified GM mass fraction value (< 0.9 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 0.3 g/kg, which is below the certified < 0.9 g/kg value. The LOD is, therefore, already a conservative estimate of the certified value and no uncertainty is assigned.

Table 8: Uncertainty budget for the mass fraction of MON 810 maize in ERM-BF413k

ERM	Certified value [g/kg]	Standard uncertainty contribution [g/kg]						Expanded uncertainty 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)	
BF413ak	**< 0.9** 1)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	**-**
BF413ck	**4.9**	0.3821	0.0554	0.0071	0.0007	0.2615	0.0262	**1.0**
BF413ek	**19.8**	0.6248	0.2218	0.0221	0.0026	0.2615	0.1050	**1.5**
BF413gk	**99**	1.9575	1.1089	0.0916	0.0105	0.2615	0.5249	**5**

1) With a 95 % probability, the certified value is below this level.
2) Standard uncertainty contribution resulting from the homogeneity assessment.
3) Standard uncertainty resulting from the stability 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 (2.1 g/kg) and of the highest water mass fraction found in any of the powders used for mixing (36.6 g/kg for the non-GM powder).
6) Standard uncertainty of the purity estimation of the non-GM base material (LOD = 0.9 g/kg), based on the half-width of the interval between 0 and 0.9 g/kg, divided by the square root of 3 (rectangular distribution).
7) Standard uncertainty of the purity estimation of the GM base material (> 97 %), based on the half-width of the interval between 97 % and 100 % divided by the square root of 3 (triangular distribution).

Table 9: Uncertainty budget for the GM DNA copy number ratio in ERM-BF413ek

ERM	Certified value [%]	Standard uncertainty contribution [%]			Expanded uncertainty U (k = 2) [%]
		u_{bb} 1)	u_{lts} 2)	u_{char} 3)	
BF413ek	**0.77**	0.0228	0.0046	0.0281	**0.08**

1) Standard uncertainty contribution resulting from the homogeneity assessment.
2) Standard uncertainty determined from the stability of ERM-BF413d during storage, extrapolated to a shelf life of 12 months.
3) Standard uncertainty of the GM DNA copy number ratio determination.

8 Metrological traceability

The ERM-BF413k series is composed of four reference materials certified for the mass fraction of event MON 810 maize seed powder. The certified values in GM mass fractions are based on gravimetric dry-mixing of non-modified maize seed powder with event MON 810 maize seed powder. The respective certified value is traceable to the International System of Units (SI). The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure. The purity of the used seeds has been taken into account when calculating the certified value.

ERM-BF413ek is additionally certified for its GM DNA copy number ratio. The certified GM DNA copy number ratio is defined as the percentage of MON 810 DNA copy numbers in relation to maize-specific DNA copy numbers calculated in terms of haploid genomes [2]. Charels et al. reported that the GM DNA copy number ratio was dependent on the real-time PCR method applied [20]. Therefore, and given the interlaboratory comparison comprising 17 accepted data sets, the certified value for the GM DNA copy number ratio is traceable to the event-specific CRL-GMFF validated rt-PCR detection method [6] calibrated with the MON 810 maize plasmid DNA CRM ERM-AD413 [18]. The measurement results from the interlaboratory comparison were shown to be independent of the DNA extraction method applied (**Section 6.2**).

9 Intended use and instructions for use

The ERM-BF413k 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.

Measurements in GM DNA copy number ratio, using ERM-BF413ek, should be performed exclusively with the event-specific MON 810 detection method ISO 21570:2005, Annex D2 [6], calibrated with the plasmid DNA calibrant ERM-AD413 [18] as the certified value is traceable to this method and calibrant. ERM-BF413ek can also be used for calibration but it is recommended to use ERM-AD413 [18] for calibration purposes because of its small stated uncertainty.

The user of the certified reference material should bear in mind that the numerical values for the GM DNA copy number ratio measured by real-time PCR differ from the certified GM mass fraction as a result of different tissues and their genetic background. Due to different DNA extraction efficiencies from GM and non-GM powders and the various tissue types it is not possible to calculate a conversion factor for the two units.

The DNA extraction yield may be lower for ERM-BF413k (and other maize CRMs from IRMM) compared to ERM-BF413 because the latter was processed differently, using grinding techniques involving liquid Nitrogen, which resulted in a finer powder.

The recommended minimum sample intake is 100 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.

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] Trapmann S., Le Guern L., Prokisch J., Robouch P., Kramer G.N., Schimmel H., Querci M., Van den Eede G., Anklam E., Pauwels J. (2001) The Certification of Reference Materials of Dry Mixed Maize Powder with different Mass Fractions of MON 810 Maize. Certified Reference Materials IRMM-413. ISBN 92-894-1978-4. EC certification report EUR 20111 EN.

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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) MON 810 maize (ERM-BF413ak, ck, ek, gk). The materials were processed and certified in 2007/2008 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), according to the principles of ISO Guide 34. The four CRMs belonging to the ERM-BF413k series replace the earlier produced series ERM-BF413 which is exhausted. The four CRMs have been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.
Hybrid seeds of GM MON 810 maize and of a comparable non-GM maize variety were dried and ground to GM and non-GM base powders. A non-GM pure material and three 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 (measurements within the scope of accreditation to ISO/IEC 17025).
European Commission Recommendation (EC) No 787/2004 advises to express the content of genetically modified food and feed as the percentage of genetically modified DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes, the so-called GM DNA copy number ratio. An interlaboratory comparison was, therefore, conducted in 2008 to certify one of the CRMs, ERM-BF413ek, additionally for the GM DNA copy number ratio.
The certified values and uncertainties of the four CRMs are as follows:

CRM	Quantity	Certified value	Uncertainty [4)]
ERM-BF413ak	Mass fraction [1)] [g/kg]	< 0.9 [3)]	-
ERM-BF413ck	Mass fraction [1)] [g/kg]	4.9	1.0
ERM-BF413ek	Mass fraction [1)] [g/kg]	19.8	1.5
	GM DNA copy number ratio [2)] [%]	0.77	0.08
ERM-BF413gk	Mass fraction [1)] [g/kg]	99	5

[1)] The certified mass fraction is based on the mass fraction of MON 810 maize seed powder mixed in non-genetically modified maize seed powder and taking into account their respective MON 810 maize purity and their water mass fraction. The certified value is traceable to the SI.
[2)] The certified GM DNA copy number ratio is the unweighted mean of 19 accepted data sets. The certified value is traceable to the real-time PCR detection method ISO 21570:2005, Annex D2 calibrated with the MON 810 maize plasmid DNA CRM ERM-AD413.
[3)] With a 95 % probability, the value of the material is below this level.
[4)] 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 MON 810 maize in food and feed. The CRMs are available in glass vials containing 1 g of dried maize powder closed under argon atmosphere. The minimum sample intake per analysis is 100 mg.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

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