Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of a MON 810 Maize Reference Material for its DNA copy number ratio

Certified Reference Material ERM®-BF413d

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Certification of a MON 810 Maize Reference Material for its DNA copy number ratio

Certified Reference Material ERM®-BF413d

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Glossary

ANOVA	analysis of variance
bp	base pair
Bt	*Bacillus thuringiensis*
cp	number of copies
CRL-GMFF	Community Reference Laboratory for GM Food and Feed
CRM	Certified Reference Material
cryIA(b)[1]	gene encoding the CryIA(b) δ-endotoxin from *Bacillus thuringiensis*
CryIA(b)[1]	δ-endotoxin from *Bacillus thuringiensis*
CTAB	cetyltrimethylammonium bromide
Ct-value	number of PCR cycles to pass a set threshold
DNA	deoxyribonucleic acid
ERM®	trademark of European Reference Materials
gDNA	genomic DNA
GM	genetically modified
GMO	genetically modified organism
hmg	high mobility group gene from *Zea mays* L. (taxon-specific gene)
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
µL	microliter
MON 810	GM *Zea mays* L. event MON 810
MS_{bb}	mean sum of squares between bottles
MS_{wb}	mean sum of squares within bottles
n	number of samples analysed
N	number of bottles analysed, number of data sets
p	probability
PCR	polymerase chain reaction
pDNA	plasmidic DNA
R^2	correlation coefficient
RM	Reference Material
RSD	relative standard deviation
rt-PCR	real-time PCR
s_{bb}	standard deviation between bottles
s	standard deviation
U	expanded uncertainty
u_{bb}	standard uncertainty related to the between-bottle heterogeneity
u_{char}	standard uncertainty introduced by the characterisation
U_{CRM}	expanded uncertainty of the certified value
u_{lts}	standard uncertainty related to the stability, estimated on the basis of a shelf life of 18 months
UV	ultra violet
P35S	P35S promoter, derived from Cauliflower Mosaic Virus
$\overline{x}$	average
$\overline{x}_i$	average obtained for data set *i*

[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.

1 Introduction

Legislation in the European Union demands the labeling of food products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs), provided the GMO has been placed on the market in accordance with Community legislation [1]. This enforces the necessity on the one hand to develop and validate reliable quantitative measurement methods and on the other hand to develop and produce reference materials (RMs) to calibrate and control the correct application of detection methods. Until recently, GMO Certified Reference Materials (CRMs) from the Institute for Reference Materials and Measurements (IRMM) have been produced by mixing genetically modified (GM) powder with non-GM powder and have been certified for their GM mass fraction.

In October 2004, the European Commission recommended 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]. As a consequence, the existing CRMs should be certified for the DNA copy number ratio. The DNA copy number ratio, expressed in %, is determined according to the following formula:

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

The CRM ERM®-BF413d, consisting of non-modified and GM MON 810 maize powder, was certified in January 2001 for the GM mass fraction [1]. In 2007, an interlaboratory comparison was conducted to certify ERM-BF413d for its DNA copy number ratio. The plasmidic DNA (pDNA) calibrant ERM®-AD413, containing MON 810 and taxon-specific DNA sequences in a 1:1 ratio [3], and the real-time polymerase chain reaction (rt-PCR) quantification method ISO 21570:2005, Annex D2 [4], validated by the Community Reference Laboratory for GM Food and Feed (CRL-GMFF), were used in this study. The DNA calibrant ERM-AD413 is a DNA solution containing approximately 2×10^6 copies per microliter (cp/µL) of a plasmid into which a 170 bp fragment of the MON 810 plant/P35S junction as well as a 351 bp fragment of the maize endogenous high mobility group gene (*hmg*) have been inserted [3]. Both fragments are specifically targeted by the event-specific MON 810 detection method used.

The certification of ERM-BF413d for its DNA copy number ratio was based on rt-PCR measurements:

- to characterise ERM-BF413d with respect to the event MON 810 DNA copy number ratio
- to assess the homogeneity, the short-term and the long-term stability of ERM-BF413d

The material ERM-BF413d is intended to be used for quality control of measurements of the MON 810 DNA copy number ratios in GM food and feed using the ERM-AD413 calibrant and the event-specific MON 810 detection method ISO 21570:2005, Annex D2 [4]. ERM-BF413d can alternatively be used for calibration but it is recommended rather to use ERM-AD413 because of the small stated uncertainty.

2 Participants

Characterisation of ERM-BF413d for its DNA copy number ratio

- Bundesinstitut für Risikobewertung (BfR) - FGr 56, Berlin, DE*
- Centre Wallon de Recherches Agronomiques (CRA-W) - Département Qualité des Productions Agricoles, Gembloux, BE*
- Eurofins Analytik GmbH - WEJ Dept Biology 135, Hamburg, DE*
- European Commission, Joint Research Centre, Institute for Health and Consumer Protection (IHCP) - Biotechnology & GMOs Unit, Ispra, IT*
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM) - RM Unit, Geel, BE*
- Finnish Customs Laboratory - Tullilaboratorio, Espoo, FI*
- Groupe d'études et de contrôle des variétés et des semences (GEVES) - BioGEVES, Surgères, FR*
- Hainaut Vigilance Sanitaire - Institut Provincial d'Information et d'Analyses Sanitaires, Mons, BE*
- Istituto Superiore di Sanità (ISS) - Centro Nazionale per la Qualità degli Alimenti e per i Rischi Alimentari - Reparto Organismi Geneticamente Modificati e Xenobiotici di origine fungina, Roma, IT
- Istituto Zooprofilattico Sperimentale Lazio e Toscana - Dipartimento di Virologia e Biotecnologie, Roma, IT*
- Korea Research Institute of Standards and Science (KRISS) - Organic and Bio Analysis Group, Daejeon, KR
- Landesamt für Umweltschutz Sachsen-Anhalt, FG 13 Gentechnisches Überwachungslabor, Halle/Saale, DE*
- National Food Research Institute (NFRI) - Molecular Engineering Lab, Tsukuba, JP
- National Institute of Biology (NIB), Ljubljana, SI*
- Nestlé Research Center Lausanne (Nestec S.A.) - Department Quality & Safety Assurance, Lausanne, CH*
- Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Lebensmittelinstitut Braunschweig, Braunschweig, DE*
- Ontario Plant Laboratories - Canadian Food Inspection Agency - Ottawa Laboratory Fallowfield, Ottawa, CA*
- Staatliches Gewerbeaufsichtsamt Hildesheim - Dez 33 Gentechnik, Hildesheim, DE*
- Umweltbundesamt Wien (UBA Wien), Vienna, AT*

Assessment of the homogeneity and the stability of ERM-BF413d

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

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

3 CRM preparation

ERM-BF413d is a gravimetrically prepared dried maize powder containing a certified mass fraction $\pm$ *U* (*k* = 2) of 10.0 $\pm$ 0.5 g/kg of the GM maize event MON 810. ERM-BF413d was processed in 2000 as one CRM of a set of six CRMs certified for their mass fraction of MON 810. For a detailed description of the processing of ERM-BF413d, the reader is referred to an earlier report [1].

4 Homogeneity

4.1 Homogeneity study

The homogeneity of the MON 810 maize DNA copy number ratio in ERM-BF413d was measured by rt-PCR, using the pDNA calibrant ERM-AD413 for calibration and the event-specific MON 810 PCR detection method ISO 21570:2005, Annex D2 [4]. The homogeneity was investigated under repeatability conditions using 15 bottles, randomly taken from the entire batch. From each bottle three replicates were analysed using a sample intake of 200 mg. Grubbs tests did not detect any outlying values related to the individual results and bottle averages (N = 15, n = 3).
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 for the results of the sample averages (n = 3). A significant trend was shown for the filling sequence. The drift in the results related to the filling sequence is covered by the uncertainty related to the between-bottle heterogeneity.

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 (n = 3) were normally distributed.

ANOVA statistics was performed (**Table 1**) to calculate the between-bottle standard deviation (s_{bb}) using the formula [9]:

$$s_{bb} = \sqrt{\frac{MS_{bb} - MS_{wb}}{n}}$$

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

The value for s_{bb} was used as an estimate for the uncertainty contribution related to the between-bottle heterogeneity (u_{bb}) and included in the calculation of the overall uncertainty of the certified value (**Section 7.3**).

Table 1: Calculation of the standard deviation related to the between-bottle heterogeneity of ERM-BF413d, analysed by rt-PCR using a sample intake of 200 mg (*N* = 15, *n* = 3)

Quantity	s_{bb} [%]	$s_{bb,rel}$ [%]
DNA copy number ratio	0.08	13.4

4.2 Minimum sample intake

A sample intake of 200 mg powder was used for DNA extraction by the GENE*Spin* method (GeneScan Analytics GmbH, Freiburg, DE) in the homogeneity study. The assumption that this amount ensures a sufficient homogeneity was investigated and proved to be correct. For the validity of this certified value, sample intakes not smaller than 200 mg have to be used.

5 Stability

5.1 Short-term stability

In order to assess whether special care must be taken during transportation, the short-term stability of ERM-BF413d was investigated using an isochronous approach [10]. The significance of the trend line was evaluated after exposure of the material for certain time periods at different temperatures. The material is considered to be stable at the given temperature if the slope of the linear regression line is not significantly different from zero at a 95 % confidence level.

Five bottles were stored at each of the Celsius temperatures 4 °C, 18 °C and 60 °C and during each of the periods 2 and 4 weeks; three samples from each bottle were analysed (N = 5, n = 3). Five reference bottles were kept at -70 °C for the duration of the study. Bottles were immediately shifted to -70 °C following 2 and 4 weeks incubation at the tested temperatures. All bottles were subsequently kept at -70 °C until they were analysed. Genomic DNA (gDNA) was extracted from the samples according to the GENE*Spin* method using a sample intake of 200 mg. The DNA concentration was estimated by UV spectrometry and by fluorometry (PicoGreen dsDNA assay kit, Molecular Probes Europe, Leiden, NL). The DNA pattern was visualised by gel electrophoresis. No substantial DNA degradation was seen in any of the samples. Each DNA extract was analysed in triplicate by event-specific rt-PCR to reveal changes in GM quantification (**Figure 1**). Single and double Grubbs tests showed one outlier (95 % confidence level) in the data from the reference samples (stored at -70 ºC). Regression analysis was performed for each of the tested temperatures to reveal any trend in GM quantity in relation to the exposure time. A *t*-test showed no sign of a trend even after storage at 60 °C for a time period of 4 weeks (95 % confidence level). Removal of the above-mentioned outlier did not change the significance of the slope of the linear regression line. It was concluded that ERM-BF413d can be shipped under ambient conditions.

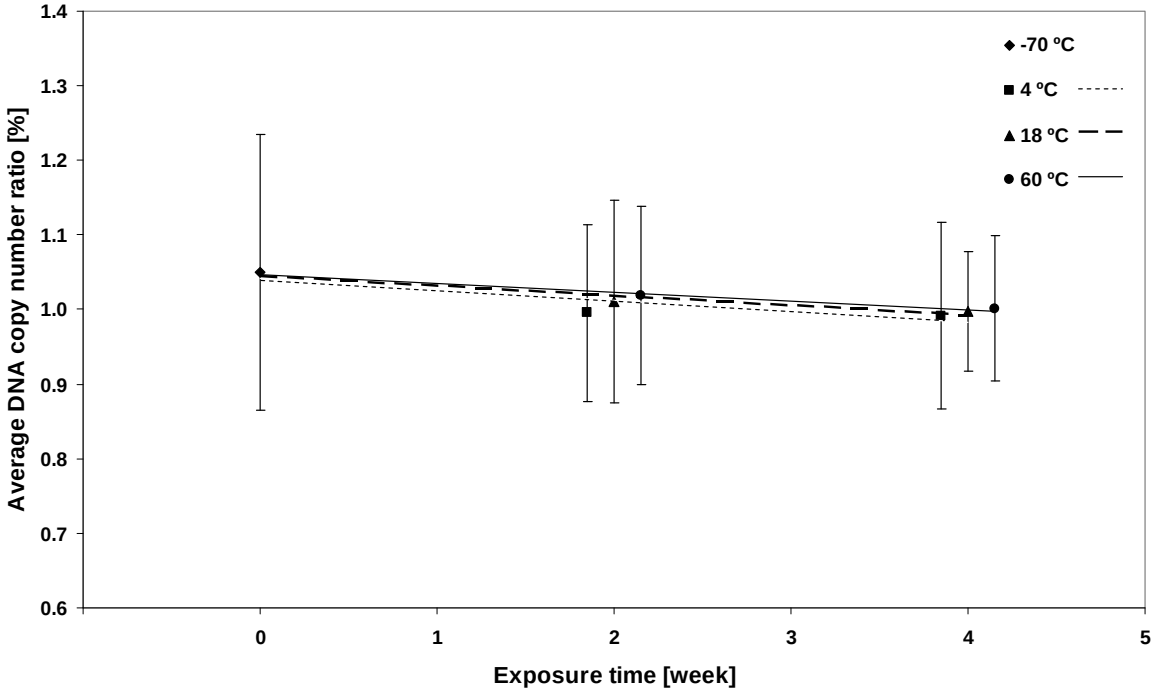

Figure 1: Short-term stability of ERM-BF413d exposed to different temperatures for 2 and 4 weeks, and analysed by event-specific rt-PCR [4]. Exposure time 0 refers to the data from the reference samples (-70 ºC). Bars indicate the average DNA copy number ratio $\bar{x}$ ± *s* for *N* = 5, *n* = 3. Rt-PCR measurements were done in triplicate on each DNA extract.

5.2 Long-term stability

The stability of the event MON 810 maize seed powder was unaffected by short-term incubation at elevated temperatures (**Section 5.1**), similarly to what was observed for other maize matrices in the past [11]. The long-term stability of ERM-BF413d has been monitored for more than 6 years, using among other quantification methods the event-specific MON 810 detection method ISO 21570:2005, Annex D2 [4] (**Figure 2**). Because the certified value is traceable to the above-mentioned rt-PCR detection method [4], it was important to take only the data from stability monitoring of ERM-BF413d to calculate the standard uncertainty contribution from the stability.

Bottles were stored at -70 ºC and 4 ºC during 67 (*N* = 1), 75 (*N* = 3) and 81 months (*N* = 1), respectively, and analysed immediately after incubation. From each bottle three DNA extractions (*n* = 3) were carried out with the Wizard Magnetic Purification System for Food (Promega Benelux, Leiden, NL) and a KingFisher magnetic particle processor (Thermo Fisher Scientific, Zellik, BE), and using a sample intake of 50 mg. The MON 810 mass fraction was measured by rt-PCR, using a MON 810 gDNA calibrant and the event-specific MON 810 PCR detection method ISO 21570:2005, Annex D2 [4]. The MON 810 mass fraction ratio $\bar{x}_{4\,°C} / \bar{x}_{-70\,°C}$ of samples stored at 4 ºC and -70 ºC was calculated.

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* = 0.19).

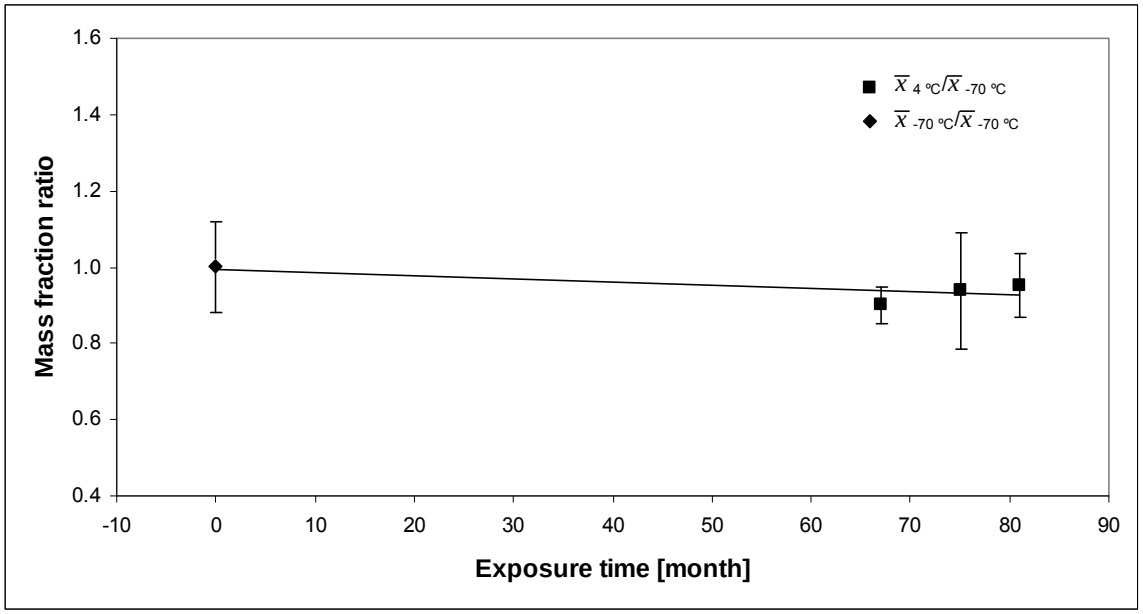

Figure 2: Long term-stability of maize ERM-BF413d stored at 4 °C for up to 81 months assessed by rt-PCR. Time point 0 shows the MON 810 mass fraction ratio of samples stored at -70 ºC $\overline{x}_{-70\,°C}/\overline{x}_{-70\,°C}$ ± *s* (*N* = 5, *n* = 3). Bars indicate the MON 810 mass fraction ratio of samples stored at 4 ºC and -70 ºC $\overline{x}_{4\,°C}/\overline{x}_{-70\,°C}$ ± *s* (*N* = 1 for 67 and 81 months and *N* = 3 for 75 months storage, *n* = 3). Rt-PCR measurements were done in triplicate on each DNA extract.

The uncertainty contribution from the long-term stability is estimated by calculating the uncertainty on the mass fraction ratio ($\overline{x}_{4\,°C}/\overline{x}_{-70\,°C}$) of samples stored at 4 ºC and -70 ºC. Accepting a shelf life of 18 months, a standard uncertainty contribution of 1.1 % for the stability was calculated (**Table 4**, **Section 7.3**). It is recommended to store the bottles at 4 ºC.
Post-certification monitoring is being carried out at regular intervals in order to further check the stability of ERM-BF413d.

6 Characterisation study

Nineteen laboratories were selected on the basis of proven experience and quality assurance systems in place. For the characterisation of ERM-BF413d with respect to the DNA copy number ratio a total of 20 data sets were analysed. Two different DNA extraction methods, a modified cetyltrimethylammonium bromide (CTAB) method [12] and the GENE*Spin* extraction method were tested, whereby 10 data sets were allocated to each method. Each participating laboratory was assigned a single data set with the exception of IRMM that performed two data sets.

Matrix CRM ERM-BF413d, all reagents, DNA extraction and PicoGreen dsDNA assay kits, DNA calibrant, and consumables were shipped to the participating laboratories on dry ice.

DNA was extracted from two bottles of ERM-BF413d using the commercial GENE*Spin* kit and a modified CTAB DNA extraction method, with the cesium chloride purification step replaced by an additional extraction with chloroform and a final ethanol precipitation. Three samples from each bottle were analysed (N = 2, n = 3) whereby all experiments had to be spread over at least two days. The DNA concentration was estimated by UV spectrometry and/or by fluorometry. The DNA concentration of each DNA extract was adjusted to 10 ng/µL with nuclease-free water and the extracts were further diluted 2, 4, 6 and 8 x to assess the possible occurrence of PCR inhibition. Undiluted and diluted samples were analysed in quadruplicate by event-specific rt-PCR using primer pairs and labelled TaqMan® probes specific for maize event MON 810 and for the maize endogenous *hmg* gene [13]. Dilution series were prepared by the participating laboratories from the pDNA calibrant ERM-AD413 in a background of ColE1 pDNA (1 ng/µL) ranging from 2 x 10^6 cp/µL to 5 cp/µL [3]. TaqMan Universal PCR experiments were carried out according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA) with all runs performed for 45 cycles.

Exclusion of data sets was done according to the technical exclusion criteria described by Charels *et al.* [13]. These include the PCR efficiency estimated on the basis of the slope of the calibration curve, the correlation coefficient of the calibration curve, PCR inhibition analyses, technical mistakes and anomalies related to the dilution of gDNA extracted from ERM-BF413d.

In accordance with the above-described exclusion criteria, data sets from three laboratories were excluded. One data set was removed because the laboratory had not strictly followed the protocol provided by IRMM concerning the preparation of a fresh dilution series of the pDNA calibrant ERM-AD413 on the second day of the experiments. Another data set was excluded because of a 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 [14]. A third data set was removed because all reported Ct-values fell beyond the linear working range of the calibration curve for the detection of the GM target.

DNA copy number ratios were determined for both combinations of DNA extraction and rt-PCR detection methods for the undiluted, 2, 4, 6 and 8 x diluted samples. The DNA copy number ratio was calculated as the average determined for undiluted, 2, 4, 6 and 8 x diluted samples, provided the results were within the linear range of the calibration curve (**Table 2**). To assess the occurrence of PCR inhibitors in the DNA extracts, the difference between the measured DNA copy number ratios of the various dilutions was compared. For each of the 102 DNA extracts (i.e. number of accepted data sets multiplied by number of DNA extractions performed or 17 x 6), this difference remained within the limits of variation for rt-PCR (RSD between 15 and 25 %) with three exceptions (RSD = 29, 40 and 41 %, respectively). Therefore, no individual measurement result was excluded on the basis of PCR inhibition.

Table 2: Average DNA copy number ratio and *s* obtained for each data set.

Data set number	DNA extraction method	DNA copy number ratio [%]	*s* [%]
1	CTAB	0.50	0.05
2	CTAB	0.59	0.17
3	CTAB	0.54	0.04
4	CTAB	0.65	0.07
5	CTAB	0.73	0.07
6	CTAB	0.42	0.14
7	CTAB	0.45	0.05
8	CTAB	0.74	0.05
9	CTAB	0.54	0.08
10	CTAB	0.69	0.16
11	GENE*Spin*	0.51	0.05
12	GENE*Spin*	0.49	0.14
13	GENE*Spin*	0.50	0.05
14	GENE*Spin*	0.49	0.03
18	GENE*Spin*	0.64	0.11
19	GENE*Spin*	0.53	0.07
20	GENE*Spin*	0.75	0.03

Table 3: Certified value ($\overline{x}$), *s* and standard uncertainty related to the characterisation (u_{char}, Section 7.3). $\overline{x}$ is calculated as the unweighted mean of means.

N	$\overline{x}$ [%]	*s* [%]	u_{char} [%]
17	0.57	0.11	0.026

In total, 17 data sets were subjected to further analyses. Scrutinising these data, no outliers were detected by Nalimov *t*-test, Dixon test and Grubbs tests (95 % confidence level). The Cochran test showed no outlying data set variances and the Bartlett test confirmed data set variances to be homogeneous. Both skewness and kurtosis tests indicate that the data are normally distributed (95 % confidence level).

In addition, 'main effects' ANOVA was carried out using the DNA copy number ratio as a dependent variable and the samples and the data set number as categorical factors. Both 'main effects' ANOVA ($p = 3.14 \times 10^{-11}$) (**Figure 3**) and a Snedecor *F*-test (95 % confidence level) revealed significant differences between data sets.

Two independent factorial ANOVA analyses were performed using as a dependent variable the DNA copy number ratio but differing in the categorical factors used. For the first analysis the DNA extraction method and samples were selected as categorical factors, whereas the DNA extraction method and day of the experiment (day 1 or day 2) were the categorical factors for the second factorial ANOVA. In both cases there was no significant influence of the DNA extraction method ($p = 0.34$ and $p = 0.32$, respectively) on the DNA copy number ratio. However, factorial ANOVA and a Snedecor *F*-test (95 % confidence level) showed significantly different DNA copy number ratios within the data sets for samples analysed on day 1 and day 2. The significance of the variation within data sets was dependent on the

DNA extraction method applied. The average DNA copy number ratio between days shows a difference for the GENE*Spin* kit (**Figure 4**). Hence, the modified CTAB DNA extraction method seems to be more robust because it gives a smaller day-to-day variation than the GENE*Spin* kit.

The DNA extraction method has no impact on the DNA copy number ratio in this study. The variation between data sets and the variation within data sets reported in this study, illustrate the importance of selecting a sufficient number of laboratories for the characterisation study.

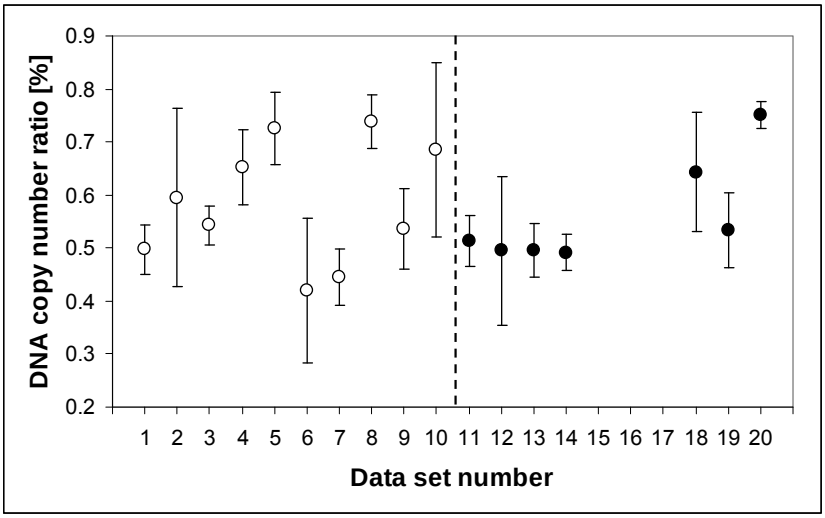

Figure 3: Differences between data sets using the CTAB (○) and GENE*Spin* (●) DNA extraction methods. Data sets 15 to 17 were excluded from further analyses (see above). Vertical bars denote *s*.

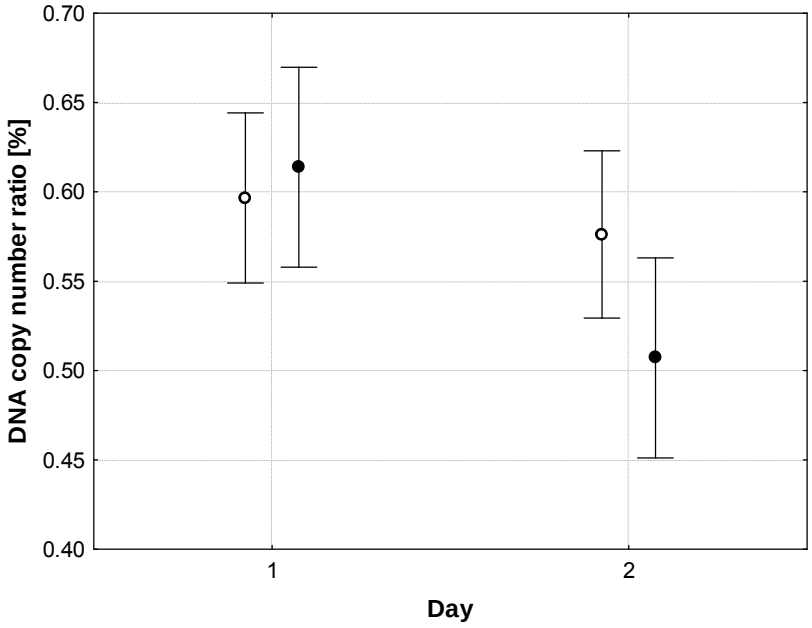

Figure 4: Influence of the CTAB (○) and GENE*Spin* (●) DNA extraction methods and day of analysis. Vertical bars denote the 95 % confidence interval.

7 Certified DNA copy number ratio and uncertainty budgets

7.1 Metrological traceability

ERM-BF413d is a RM which was first certified for the mass fraction of MON 810 maize powder [1]. This material is now additionally certified for its DNA copy number ratio. The certified 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]. During the feasibility study it was shown that the DNA copy number ratio is depending on the rt-PCR method applied [13]. Therefore, and given the interlaboratory comparison comprising 17 accepted data sets, the certified value for the DNA copy number ratio is traceable to the event-specific CRL-GMFF validated rt-PCR detection method [4] calibrated with the MON 810 maize pDNA CRM ERM-AD413 [3]. The measurement results from the interlaboratory comparison were shown to be independent of the DNA extraction method applied (**Section 6**).

7.2 Certified value

The certified value is based on the MON 810 DNA copy numbers and maize-specific DNA copy numbers. The DNA copy number ratio obtained for each data set, and expressed in %, is calculated as follows:

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

Because the data are normally distributed (**Section 6**), the certified value ($\overline{x}$) is calculated as the unweighted mean of data set means.

$$\overline{x} = \frac{\sum_{i=1}^{N} \overline{x}_i}{N}$$

where $\overline{x}$ = certified value

$\overline{x}_i$ = average DNA copy number ratio obtained for data set *i*

N = number of data sets

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 uncertainty from the characterisation has been assessed during the interlaboratory comparison by estimating the RSD of the normally distributed data. The standard uncertainty (u_{char}) related to the characterisation is calculated using the formula:

$$u_{char} = \frac{RSD}{\sqrt{N}}$$

where RSD = relative standard deviation

N = number of data sets

The uncertainty introduced by the inhomogeneity at 200 mg level has been estimated on the basis of the heterogeneity of a normally distributed population. The uncertainty contribution from the stability (u_{lts}) has been estimated on the basis of rt-PCR results following long-term storage of ERM-BF413d (**Figure 2**). A coverage factor of 2 (k = 2) was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % (**Table 4**).

The standard uncertainty contribution introduced by the inhomogeneity is larger than the standard uncertainty contributions from the characterisation and from the stability of the maize powders (**Table 4**).

Table 4: Uncertainty budget for the DNA copy number ratio of MON 810 maize in ERM-BF413d expressed in %

CRM certified value [%]	**Standard uncertainty contributions [%]**			**Expanded uncertainty U_{CRM} (k = 2) [%]**
	u_{bb} [1)]	u_{lts} [2)]	u_{char} [3)]	U_{CRM}
0.57	0.08	0.011	0.026	0.17

[1)] Standard uncertainty introduced by the inhomogeneity at 200 mg level.
[2)] Standard uncertainty related to the stability, estimated on the basis of a shelf life of 18 months.
[3)] Standard uncertainty introduced by the characterisation.

8 Instructions for use

The material ERM-BF413d is certified for both its mass fraction of the GM event MON 810 [1] and the DNA copy number ratio when applying event-specific MON 810 rt-PCR [this report].

The certified DNA copy number ratio is intended to be used for quality control of measurements of the MON 810 DNA copy number ratios in GM food and feed. Genomic DNA may be extracted from the powder applying a validated method of choice for further use in rt-PCR measurements. Experiments should be performed exclusively with the event-specific MON 810 detection method ISO 21570:2005, Annex D2 [4], calibrated with the pDNA calibrant ERM-AD413 [3] as the certified value is traceable to this method and calibrant. ERM-BF413d can also be used for calibration but it is recommended rather to use ERM-AD413 because of the small stated uncertainty.

9 Acknowledgements

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10 References

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[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.

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Abstract

This report describes the certification of the Certified Reference Material ERM®-BF413d, composed of conventional and genetically modified MON 810 maize powder, for its DNA copy number ratio.

The genetically modified *Zea mays* L. line MON 810 (Yieldguard®) was genetically engineered to resist the European corn borer (*Ostrinia nubilalis*) and is commercialised by Monsanto Company. This line was developed by introducing the *cryIA(b)* gene from the common soil bacterium *Bacillus thuringiensis* (Bt) into the maize cultivar Hi-II by particle acceleration (biolistic) transformation. The *cryIA(b)* gene produces the insect control protein CryIA(b), a δ-endotoxin.

ERM-BF413d is part of a set of maize CRMs containing different mass fractions of genetically modified MON 810 maize. The CRM was processed and originally certified for its mass fraction by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements, RM Unit, Geel, Belgium [1].

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 [2], the so-called DNA copy number ratio. An interlaboratory comparison was, therefore, conducted in 2007 to certify the existing CRM for the DNA copy number ratio. The CRM is intended for quality control of measurements of the MON 810 DNA copy number ratios in genetically modified food and feed, using the plasmidic DNA ERM®-AD413 [3] for calibration and the event-specific MON 810 detection method ISO 21570:2005, Annex D2 [4]. ERM-AD413 contains a 170 bp fragment of the MON 810 plant/P35S junction [5] and a 351 bp fragment of the maize endogenous high mobility group gene (*hmg*) [6], specific for MON 810 maize and the maize taxon, respectively.

The Certified Reference Material ERM-BF413d is available in glass bottles containing 1 g of maize powder, closed under argon atmosphere and was certified to contain the following DNA copy number ratio:

CRM	**Certified value MON 810 DNA copy number ratio [1)] [%]**	**Uncertainty [2)] [%]**
ERM-BF413d	0.57	0.17

[1)] The certified DNA copy number ratio is the unweighted mean of 17 accepted data sets. The certified value is traceable to the real-time PCR detection method ISO 21570:2005, Annex D2 [4] calibrated with the MON 810 maize plasmidic DNA Certified Reference Material ERM-AD413 [3].
[2)] The certified uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor $k = 2$, corresponding to a level of confidence of about 95 % [7].

The minimum amount of sample to be used is 200 mg.

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