

CERTIFICATION REPORT

The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 1507 Maize Certified Reference Materials ERM®-BF418

**(ERM®-BF418a / ERM®-BF418b / ERM®-BF418c /
ERM®-BF418d)**

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

European Commission

Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements

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Certified Reference Materials ERM[®]-BF418

**(ERM[®]-BF418a / ERM[®]-BF418b / ERM[®]-BF418c /
ERM[®]-BF418d)**

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ADDENDUM

Certification report ERM-BF418

EUR 21689 EN

Additional information

ANNEX

Use of ERM-BF418 for measurement results based on copy number ratios

For the preparation of the CRMs ERM-BF418, Pioneer Hi-Bred International (Johnston, IA, USA) supplied seeds of non-modified maize and GM 1507 maize to the IRMM. According to information provided by Pioneer, the GM 1507 maize seeds (unique identifier DAS-Ø15Ø7-1) are hybrid seeds, where the GM donor of the GM event is the female parent.

ERM-BF418 has been certified for its GM mass fraction and not for its GM copy number ratio. If users intend to use ERM-BF418 for GM measurement results expressed in copy number ratios, the estimation of the copy number ratio and the related uncertainty needs to be carried out according to the principles explained in ERM Application note 4 [2].

For the estimation of the copy number ratio the measurement unit of the certified value given in g/kg to the measurement unit '1' for ratios, expressed in percent, is required. Furthermore, the hybrid status of the seeds used for the production of the CRMs needs to be considered as well as the DNA extractability of the non GM and GM seed powder.

This estimation is linked to additional standard uncertainties, which need to be taken into consideration in the combined expanded uncertainty. These additional standard uncertainties refer to:

- (1) Measurement uncertainty of the quantification method
- (2) Possible variation of the maize genome size [3]
- (3) Possible effect caused by endoreduplication
- (4) Possible effect caused by the GM trait introduction of the maize event.

As the GM trait introduction via the female parent could not be verified by IRMM it is strongly recommended to take both possibilities of the GM trait introduction (introduction via the female or male parent) into consideration when calculating the combined expanded uncertainty. On the other hand it is reasonable to consider the maize seeds used for the production of the matrix to be hybrids into which the GM trait has been introduced by one parent.

The principles of the approach to estimate the copy number ratio value and its expanded combined uncertainty of a mass fraction certified maize CRM are outlined in ERM Application note 4 [2].

References

- [1] Trapmann S, Conneely P, Contreras M, Corbisier P, Gancberg D, Hannes E, Gioria S, Muñoz-Pineiro A, Van Nyen M, Schimmel H, Szilágyi S, Emons E (2005) The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 1507 Maize - Certified Reference Materials ERM®-BF418, EC certification report EUR 21689 EN, ISBN 92-894-9748-3
- [2] Application note 4: European Reference Materials - Use of certified reference materials for the quantification of GMO in food and feed.
http://www.erm-crm.org/html/ERM_products/application_notes/application_note_4/index.htm
- [3] Poggio L, Rosato M, Chiavarino AM, Naranjo CA 1998: Genome Size and Environmental Correlations in Maize (*Zea mays* ssp. *mays*, Poaceae), *Annals of Botany* 82, 107-115

SUMMARY

This report describes the preparation and certification of dry-mixed maize powder Certified Reference Materials (CRMs) with different mass fractions of genetically modified (GM) 1507 maize powder (CRMs ERM-BF418a, ERM-BF418b, ERM-BF418c and ERM-BF418d). The CRMs were processed in 2004 and certified in 2005 by the European Commission, Directorate General Joint Research Centre, the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium.

Seeds of non-modified maize and 1507 maize, both supplied by Pioneer Hi-Bred International (Johnston, IA, USA), were rinsed with demineralised water, drained and dried at 30 °C in order to minimise dust contamination from other crops. After a two step grinding process, transforming the seeds into a non-modified maize powder and a 1507 GM maize powder, the CRMs were gravimetrically prepared and homogenised by turbula- and dry-mixing.

The CRMs are intended for the quality control and calibration of methods for the detection and quantification of genetically modified food and feed. The 1507 mass fractions of ERM-BF418 were verified with the help of a DNA-based detection method. The CRMs are available in glass bottles containing 1 g of maize powder closed under argon atmosphere.

The four CRMs belonging to the set ERM-BF418 were certified to contain the following 1507 mass fractions:

CRM	Certified value 1507 mass fraction ¹⁾ [g/kg]	Uncertainty ²⁾ [g/kg]
ERM-BF418a	< 0.5	not applicable
ERM-BF418b	1.0	-0.2; +0.6
ERM-BF418c	9.9	-0.6; +0.8
ERM-BF418d	98.6	-1.7; +2.0

¹⁾ The certified value is based on the mass fraction of dried non-genetically modified powder and dried genetically modified powder mixed and corrected for the water content. The certified value is traceable to the SI.

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 %.

The minimum sample intake recommended for analysis is 100 mg.

GLOSSARY

\bar{x}	average
CRM	Certified Reference Material
CTAB	cetyltrimethylammonium bromide
Ct-value	number of PCR cycles to pass a set threshold
CV	coefficient of variation
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
FAM	6-FAM TM fluorescent dye
gDNA	genomic DNA
GM	genetically modified
GMO	genetically modified organism
HMGa	high mobility group protein A gene from <i>Zea mays</i>
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
KFT	Karl Fischer titration
LOD	limit of detection
LOQ	limit of quantification
MON 863	GM maize event MON 863
n	number of samples analysed
NAA	neutron activation analysis
PCR	polymerase chain reaction
PSA	particle size analysis
R^2	coefficient of correlation
rt-PCR	real-time PCR
s	standard deviation
SI	International System of Units
U	expanded uncertainty
u	standard uncertainty
UV	ultra-violet
1507	GM maize event 1507

Table of contents

1 INTRODUCTION.....	5
2 CRM PREPARATION.....	5
2.1 CHARACTERISATION OF THE BASE MATERIALS	5
2.2 PROCESSING OF THE GROUND BASE MATERIALS.....	8
2.3 GRAVIMETRICAL PREPARATION OF THE GM MIXTURES	9
2.4 BOTTLING.....	9
2.5 PROCESSING CONTROL.....	9
3 HOMOGENEITY	11
3.1 HOMOGENEITY STUDY AND HOMOGENEITY ASSESSMENT	11
3.2 MINIMUM SAMPLE INTAKE FOR ANALYSIS	12
4 STABILITY.....	12
4.1 SHORT-TERM STABILITY	12
4.2 LONG-TERM STABILITY	13
5 CERTIFIED MASS FRACTIONS AND UNCERTAINTY BUDGETS.....	13
5.1 TRACEABILITY.....	13
5.2 CERTIFIED VALUE	14
5.3 UNCERTAINTY BUDGET.....	14
6 VERIFICATION OF 1507 MAIZE MIXTURES.....	15
REFERENCES AND ACKNOWLEDGEMENTS	17
REFERENCES.....	17
ACKNOWLEDGEMENTS	18
ANNEX.....	19

1 Introduction

Legislation in the European Union demands the labelling of food and feed products consisting of or containing more than 0.9 % genetically modified organisms (GMOs), 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 detection methods and on the other hand to develop and produce reference materials to calibrate and control the correct application of detection methods. Therefore, mixtures of genetically modified (GM) and non-GM powders have been prepared and certified as Certified Reference Materials (CRMs).

A set of CRMs of maize powder with different mass fractions of dried GM maize powder of the transformation event 1507 (< 0.5, 1.0, 9.9, 98.6 g/kg maize) was processed and certified by IRMM. The four CRMs (ERM-BF418a, ERM-BF418b, ERM-BF418c and ERM-BF418d) are available from IRMM and its authorised distributors [2]. According to European Commission regulation (EC) No 65/2004 [3] the event 1507 maize corresponds to the unique identifier DAS-Ø15Ø7-1.

ERM-BF418 has been produced by means of dry-mixing techniques in order to minimise DNA and protein degradation during the processing.

2 CRM preparation

2.1 Characterisation of the base materials

For the preparation of the CRMs, Pioneer Hi-Bred International (Johnston, IA, USA) supplied seeds of non-modified maize and GM 1507 maize to the IRMM. 50 kg of non-modified maize and 10 kg of 1507 maize were used for the processing of ERM-BF418.

The purity and genetic composition of these batches were verified at IRMM with the help of genomic DNA (gDNA) extracted from leaves of seedlings. Seeds of each batch ($n = 52$) were randomly chosen and allowed to germinate. Genomic DNA was extracted from pieces of the young leaves with a mass of approximately 110 mg using the DNeasy® Plant Mini kit (Qiagen, Hilden, DE). The extracted DNA was analysed on a 1 % agarose gel (10 g/L) and quantified using the PicoGreen® dsDNA quantification kit (Molecular Probes Europe, Leiden, NL). The average DNA yield \pm s per 100 mg wet tissue was 5.1 ± 0.9 µg for the GM tissue and 4.8 ± 1.3 µg for the non-GM tissue.

Detection by rt-PCR was performed at IRMM following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA). Primer pairs specific for the event 1507 and the HMGa endogenous maize gene have been used together with TaqMan probes labelled with FAM. The threshold cycle values (Ct-value) determined for the 52 GM plants were compared to a calibration curve obtained from dilution series of gDNA extracted from pure 1507 powder. All GMO plants tested positive for 1507 and the measured average mass fraction \pm s of GM 1507 maize, estimated via the transgenic copy number measured relative to the

copy number of an endogenous gene, was 722 ± 97 g/kg ($n = 52$) ¹⁾. All non-GMO plants were tested in the same way and appeared negative for the event 1507 (Table 1).

Table 1: Purity test and genetic composition of the GM and non-GM seed batches used for the production of ERM-BF418

Batch	PCR method performed and primers used ¹⁾	Number of plants tested	Number of 1507 positives	Number of 1507 negatives
Non-GM	event-specific real-time PCR	52	0	52
GM	event-specific real-time PCR	52	52	0

¹⁾ Primer sequences of the event-specific 1507 method have been provided by Pioneer [4] and can be found in the Annex.

Additionally the purity of the ground non-GM base material was tested at IRMM. The analysis of randomly selected seeds and subsequent analysis of the powder (five DNA extractions from 100 mg powder each) indicated that no GM contamination was detected in the non-GM lot, i.e. the values obtained were all below the detection limit (LOD) of the rt-PCR method applied (Table 2).

Within the frame of an in-house validation of the method the LOD and the limit of quantification (LOQ) were assessed. The LOD was calculated as $(3.3 \cdot s)/b$, with s representing the standard deviation of a defined GM mass fraction and b the slope of the calibration curve. This defined GM mass fraction was taken as the lowest GM mass fraction for which the amplification efficiency was optimal. The efficiency of the amplification was determined based on the slope of the regression line between the GM mass fraction and the Ct-values, which should not be lower than the theoretical value of 3.322. The LOQ was calculated as $(10 \cdot s)/b$.

LOD and LOQ have been established by dilution of DNA extracted from pure GM 1507 powder in nuclease free water and were found to be 0.4 g/kg and 1.1 g/kg, respectively.

¹⁾ Due to the calibration with powders produced from seeds (and the genetic composition of the various tissue types), the rt-PCR results obtained for the gDNA extracted from plants can deviate considerably from pure GM powder.

Table 2: Quantification of GM 1507 contamination in the non-GM base material by event-specific rt-PCR using 100 mg sample intakes

Non-GM base material	Number of DNA extractions ²⁾	Mass fraction GM contamination ³⁾
	<i>n</i>	[g/kg]
Event-specific rt-PCR ¹⁾	5	< 0.4

¹⁾ Primer sequences of the event-specific 1507 method have been provided by Pioneer [4] and can be found in the Annex.

²⁾ Each rt-PCR analysis was carried out in triplicate.

³⁾ The measured mass fraction is below the calculated LOD = 0.4 g/kg.

In order to verify that the extractable DNA mass fraction in the GM and in the non-GM base materials is the same, the DNA was extracted from the twice ground powders (as described in section 2.2) using the CTAB method [5]. The DNA was afterwards quantified with PicoGreen (Molecular Probes Europe, Leiden, NL) in a spectrofluorometer (FLUOstar Galaxy, BMG LABTECH GmbH, Offenburg, DE) [6]. The ratio between the extractable DNA mass fraction of the two materials was calculated as:

$$\frac{\text{Extractable mass of DNA in 100 mg 1507 maize powder}}{\text{Extractable mass of DNA in 100 mg non - GM maize powder}}$$

A difference in DNA extractability between the two base materials was observed (Table 3). This difference proved to be significant at a 95 % confidence level. The user of the certified reference material should bear in mind that different extraction efficiencies of GM and non-GM powders will influence the GM mass fractions measured by rt-PCR.

The total DNA content of both powders was investigated employing a slight modification of the classical fractionation method developed initially by Ogur & Rosen [7]. Following the sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds and acidic digestion of the DNA fractions with 1 mol/L perchloric acid, the amount of DNA was measured by a colorimetric reaction with diphenylamine, a specific reagent for 2-deoxyriboses linked to purine nucleobases [7, 8]. Using the modified method a DNA ratio around 1 was found, indicating that the total DNA content of both materials was the same (Table 4).

Table 3: Ratio of extractable DNA of GM and non-GM ground base material as determined by PicoGreen in a spectrophotometer [6]

Extraction Method	<i>n</i>	Mass fraction ratio \pm s
CTAB [5]	10	0.7 ± 0.3

Table 4: Ratio of the total DNA content of GM and non-GM ground base material determined by a modified Ogur & Rosen method [7]

Extraction method	<i>n</i>	Mass fraction ratio \pm s
Modified Ogur & Rosen	9	1.1 ± 0.1

2.2 Processing of the ground base materials

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

The seeds used for processing were rinsed in demineralised water, drained, and dried under vacuum at 30 °C for a minimum of 20 hours. This treatment led to a water mass fraction loss of approximately 20 g/kg. The dried seeds were then ground using a high impact mill with a triangular ribbed open grinding track in order to obtain the ground base material. The high impact mill was flushed with nitrogen gas throughout the milling process. An additional vacuum drying at 30 °C for a minimum of 20 hours was carried out to further reduce the water of the once ground base material with a mass fraction of approximately 80 g/kg. For the second grinding step a sieve insert was used with 0.5 mm mesh width. Slow feeding of the mill ensured that the whole base material passed the sieve and that no selective grinding occurred. During grinding caution was taken to avoid that the material was exposed to temperatures above 40 °C.

The ground base material was mixed in a turbula mixer for 30 minutes to improve equal distribution of the different parts of the maize kernels separated by the milling process. Particle size analysis showed that both ground base materials had similar particle size distributions. An additional vacuum drying at 30 °C for a minimum of 20 hours was carried out after the second grinding, leading to a water mass fraction loss of approximately 20 g/kg. Prior to gravimetric preparation of the GM and non-

GM mixtures by dry-mixing both twice-ground base materials had a water mass fraction of approximately 12 g/kg.

2.3 Gravimetical preparation of the GM mixtures

The twice-ground base materials were used to produce powder mixtures containing mass fractions of 1507 maize powder at nominal levels of 0, 1, 10 and 100 g/kg. Prior to the dry-mixing, the mass fractions of water in the ground GM and non-GM base materials were determined in triplicate by volumetric Karl Fischer titration (KFT, Metrohm, Berchem, BE) in order to correct for the water content of the ground base material. The mixture for the nominal mass fraction of 100 g/kg was produced first by mixing pure GM with non-GM ground base material. All lower mass fractions were achieved by further dilution of the 100 g/kg GM powder with non-GM maize powder. Ground base materials were weighed using a calibrated balance. The powders were in a first step manually pre-mixed in a container and afterwards turbula mixed. The whole material was then transferred into a dry-mixing device and mixed for 2 min.

2.4 Bottling

The dry-mixed powders were bottled in cleaned 10-mL brown glass vials using an automatic filling device. The first 30 filled bottles of each batch were discarded as an additional precaution against carry-over contamination. Rubber stoppers were automatically placed on the bottle neck. Before final closure of the vials the air was evacuated in a freeze-drier and replaced with argon. The vials were closed with the help of a hydraulic device in the freeze-drier and then sealed with aluminium caps to prevent unintended opening during storage and transport. Colour-coded caps were used for easy identification of the different GM levels: nominal 0 g/kg - silver, nominal 1 g/kg - yellow, nominal 10 g/kg - red and nominal 100 g/kg - brown.

2.5 Processing control

The remaining mass fraction of water was determined by volumetric KFT in ten randomly selected bottles from each of the powder mixtures and typically amounted to values in the interval of 10 to 12 g/kg (Table 5).

Five randomly selected bottles from each of the powder mixtures were used for particle size measurements with a particle size analyser based on laser diffraction (PSA, Sympatec, Clausthal-Zellerfeld, DE). The powders had a maximum particle size below 735 μm (Table 6) and an average particle size around 113 μm .

Additionally, a sieving test was carried out following ISO 3310-1 using sieves with meshes of 90, 125, 180, 250, 500 and 710 μm (Table 7). For sieving analysis the content of ten randomly selected bottles from each of the powder mixtures was merged to reach the required sample intake of 10 g. It can be concluded from Table 7 that the average particle size determined by sieving test and by PSA showed similar results. The confirmed average particle size of 113 μm was used for the

calculation of the minimum sample intake (section 3.2) and the calculation of the uncertainty budget (section 5.3).

Table 5: Water mass fraction of the four CRMs

CRM	Water mass fraction [g/kg]		
	<i>n</i>	\bar{x}	<i>s</i>
ERM-BF418a	10	11.4	2.4
ERM-BF418b	10	11.3	2.7
ERM-BF418c	10	11.4	2.4
ERM-BF418d	10	10.4	2.1

Table 6: Particle size distribution of the CRMs, determined by laser light diffraction (*n* = 5)

CRM	Mass fraction of particle according to their size $\bar{x} \pm s$ [%]					
	Size < 90 μm	Size < 125 μm	Size < 180 μm	Size < 255 μm	Size < 515 μm	Size < 735 μm
ERM-BF418a	42 \pm 6	52 \pm 7	66 \pm 10	82 \pm 11	91 \pm 12	100 \pm 0
ERM-BF418b	42 \pm 5	51 \pm 6	64 \pm 7	80 \pm 8	99 \pm 2	100 \pm 0
ERM-BF418c	46 \pm 9	55 \pm 10	69 \pm 12	84 \pm 10	100 \pm 1	100 \pm 0
ERM-BF418d	48 \pm 5	58 \pm 6	73 \pm 7	90 \pm 7	100 \pm 0	100 \pm 0

Table 7: Particle size distribution of the CRMs, determined by sieving test (*n* = 1) using a sample intake of 10 g

CRM	Mass fraction of particle according to their size [%]					
	Size < 90 μm	Size < 125 μm	Size < 180 μm	Size < 250 μm	Size < 500 μm	Size < 710 μm
ERM-BF418a	29	41	53	69	100	100
ERM-BF418b	25	40	53	69	100	100
ERM-BF418c	18	39	53	59	100	100
ERM-BF418d	17	39	53	68	100	100

3 Homogeneity

3.1 Homogeneity study and homogeneity assessment

In order to ensure that the prepared CRMs are sufficiently homogenous two strategies were followed. Prior to the production of ERM-BF418 a study was carried out using other maize powders [9] produced in the same way as the maize powders used for the preparation of ERM-BF418. In this homogeneity study for maize powders Au-spiked non-GM maize powder was dry-mixed with non-spiked non-GM maize powder and the homogeneity evaluated on the distribution of Au. Beside this study, the homogeneity of the produced 1507 GM maize mixtures was investigated using rt-PCR.

All materials used in the homogeneity study were processed in the same way as described for the ground base materials in section 2.2. A part of the maize powder was spiked with Au. Afterwards a 100 g/kg mass dilution was produced and further diluted twice to reach mass fractions of 10 and 1 g/kg Au-spiked in non-spiked maize. The Au concentrations of the three mixtures were determined with the help of neutron activation analysis (NAA). The results showed the homogeneity of the dry-mixed maize powder at a sample intake level of 50 mg (Table 8) and confirmed the adequacy of the dry-mixing technology for the preparation of maize mixtures with different GM mass fractions.

Table 8: Homogeneity study on dry-mixed Au-spiked maize powder with non-spiked maize powder; results of Au determination by neutron activation analysis (NAA) with a sample intake of 50 mg ($n = 6$) [9]

Material	Mixture parts [g]		Results NAA	
	Au-spiked	Non-spiked	Au mass fraction [$\mu\text{g/g}$]	CV [%]
Au-spiked maize	1000	0	1300	3.5
Non-spiked maize	0	1000	0.005	10.0
100 g/kg mixture	100	900	132	5.5
10 g/kg mixture	10	990	12.5	4.8
1 g/kg mixture	1	999	1.24	14.6

Additionally the homogeneity of ERM-BF418 with respect to the 1507 maize mass fractions was measured by rt-PCR. In order to determine the between-bottle variation, the within-bottle variation and the maximum hidden heterogeneity of CRM ERM-BF418, five event-specific PCR measurements on five randomly selected bottles were carried out. For ERM-BF418b (nominal 1 g/kg) a relative between bottle standard deviation of 13.1 % was found. For ERM-BF418c (nominal 10 g/kg) and ERM-BF418d (nominal 100 g/kg) the relative between-bottle standard deviation was much lower than the method repeatability and it could be concluded that it was smaller than 4.9 % and 2.5 %, respectively. Comparison of the experimental data

obtained during this homogeneity testing confirmed that the approach chosen for the estimation of the inhomogeneity uncertainty contribution (Table 9) was valid.

3.2 Minimum sample intake for analysis

The mass density of the maize powder was established by tap-density measurements, carried out similar to the procedure described in [10]. Taking into account the mass density (0.92 g/mL) and the particle size distribution (average particle size 113 μm), it was estimated that the number of particles in a 100 mg sample is larger than $8 \cdot 10^4$. Consequently 100 mg of ERM-BF418b (nominal 1 g/kg) should contain around 142 GM particles. On this basis uncertainties due to sample inhomogeneity were estimated (section 5.3). Referring to the particle size distributions it is advised to use sample intakes not smaller than 100 mg.

4 Stability

4.1 Short-term stability

In order to assess whether special care must be taken during transportation, the short-term stability of dried maize powder was investigated. It can be assumed that dried maize powder produced and stored under the same conditions behaves similar. Therefore, data obtained during the short-term stability testing of ERM-BF416 certified for its MON 863 maize GM content were accepted for the short-term stability of ERM-BF418.

During the short-term stability study an isochronous approach [11] was applied and bottles, closed under argon and containing a nominal GM mass fraction of 10 g/kg were exposed to 60 °C for 2 and 8 weeks. The DNA integrity of the samples was analysed by gel electrophoresis, the extractable DNA content was determined by UV spectrometry, and the relative concentration of the transgenic sequence was verified by rt-PCR. The results were compared to results obtained for samples stored at a reference temperature of -70 °C.

UV measurements and rt-PCR data confirmed that samples can be exposed for 2-3 weeks to temperatures of 60 °C. Moreover, no DNA degradation was observed on the gels for any of the samples. Dried maize powder can therefore be shipped under ambient conditions [9]. Figure 1 summarizes the UV and rt-PCR data obtained during the study of 8 weeks.

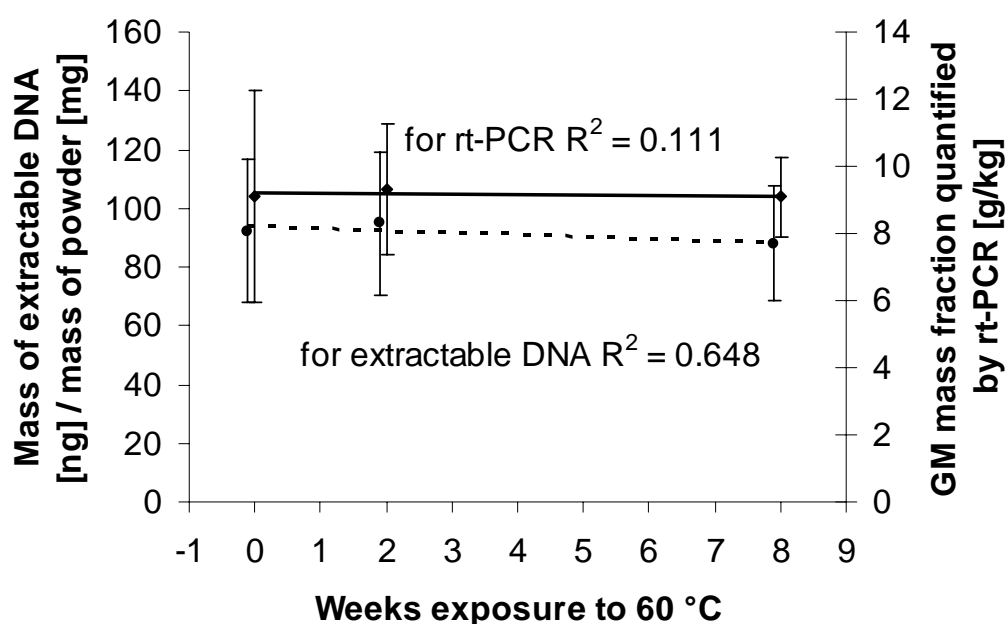


Figure 1: Short term-stability at 60 °C measured by rt-PCR (♦, regression line = full) and extractable DNA (•, regression line = dotted). The error bars indicate s for $n = 15$ [9].

4.2 Long-term stability

Earlier productions of wet-mixed maize GMO CRMs proved to have a long-term stability of at least 3.5 years provided the dried powder was stored at 4 °C in the dark and under argon. It is assumed that the stability of dry-mixed powders is higher. A post-certification monitoring is being carried out at 6-months intervals in order to monitor the stability of ERM-BF418.

5 Certified mass fractions and uncertainty budgets

5.1 Traceability

ERM-BF418a, ERM-BF418b, ERM-BF418c and ERM-BF418d form a set of four reference materials certified for the mass fraction of 1507 maize powder. The certified mass fractions are based on gravimetric dry-mixing of non-modified maize powder with 1507 maize powder.

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

It must, however, be emphasised that the mass fraction DNA / dry powder in different lots of maize kernels cannot be determined with high precision due to the relatively large uncertainty inherent to quantification of the DNA content. Moreover, the ratio between transgenic DNA and endogenous (target taxon specific) DNA in

the reference materials may significantly deviate from the certified powder mass fraction values due to the genetic composition of the maize tissues.

Furthermore, the user of the certified reference material should bear in mind that different efficiencies of the DNA extraction from GM and non-GM powders, if occurring, influence the GM concentration measured by rt-PCR.

5.2 Certified value

The certified value is based on the masses of dried genetically modified powder and dried non-genetically modified powder used in the gravimetical preparation. The masses are corrected for their water content and the purity estimates. The GM mass fraction is calculated as:

$$\frac{\text{Corrected mass GM powder}}{\text{Corrected mass GM powder} + \text{corrected mass non - GM powder}}$$

5.3 Uncertainty budget

Well controlled production techniques in combination with sound purity controls of the GM and non-GM seeds and the derived base materials allow certifying the GM mass fractions in the CRMs with relatively low uncertainties (Table 9).

The combined uncertainty of the certified value comprises the uncertainties introduced due to the weighing procedure, the water mass fraction determination, the inhomogeneity at the recommended sample intake of 100 mg, and the purity of non-GM and GM base material. The uncertainty contributions of the purity of the two base materials have only been considered for the combined 'plus' uncertainty. Even in case of a GM contamination in the non-GM material the GM mass fraction can only increase, but can never decrease. For the purity of the GM base material the genetic identity of randomly selected kernels has been checked (see section 2.1). No evidence could be found that non-GMO seeds were among the GMO seeds. Based on statistics it could be concluded that the purity is higher than 97.12 %. As no negative seeds were found it is more likely that the true value of the GM base material is closer to 100 %. For the given reasons the uncertainty contribution of the two base materials is not contributing to the 'combined minus' uncertainty. The uncertainty introduced by the inhomogeneity has been estimated on the basis of the heterogeneity of a Poisson distributed sample. The uncertainty contribution of the stability testing has not been considered in the uncertainty budget due to the limited method repeatability of rt-PCR and the lack of alternative methods.

A coverage factor of 2 was used to calculate the expanded uncertainty with an approximate confidence interval of 95 % (Table 9).

Table 9: Uncertainty budget for the mass fraction of 1507 maize in ERM-BF418 in g/kg

CRM value		Standard uncertainty contributions					Expanded uncertainty $k = 2$ $u_1 - u_3$	Expanded uncertainty $k = 2$ $u_1 - u_5$
		u_1 ¹⁾	u_2 ²⁾	u_3 ³⁾	u_4 ⁴⁾	u_5 ⁵⁾	U_{1-3}	U_{1-5}
ERM-BF418a	< 0.5	-	-	-	-	-	-	-
ERM-BF418b	1.0	0.0022	0.0015	0.0828	0.2450	0.0040	0.2	0.6
ERM-BF418c	9.9	0.0179	0.0125	0.2618	0.2450	0.0402	0.6	0.8
ERM-BF418d	98.6	0.1262	0.0887	0.8277	0.2450	0.4024	1.7	2.0

¹⁾ Uncertainty introduced by the mass determination (mainly based on the uncertainty of the balance).

²⁾ Uncertainty introduced by the dilution technique. For ERM-BF418b three dilution steps, for ERM-BF418c two dilution steps and for ERM-BF418d one dilution step were taken into consideration (average of the standard deviation of the water content was 0.9 g/kg).

³⁾ Uncertainty introduced by the inhomogeneity at 100 mg level (average particle size of 113 μm , mass density of 0.92 g/mL).

⁴⁾ Uncertainty introduced by the purity of non-GM base material.

⁵⁾ Uncertainty introduced by the purity of GM base material.

6 Verification of 1507 maize mixtures

The GM mass fractions of all four CRMs were verified using an event-specific rt-PCR method. During in-house validation, the LOD and LOQ of the method have been found to be 0.4 g/kg and 1.1 g/kg, respectively (for details see section 2.1). The results obtained can be found in Table 10.

Table 10: Quantification by event-specific 1507 real-time PCR [4]. DNA was extracted from 100 mg powder sample intakes using the CTAB method [5]

CRM	Certified 1507 mass fraction	Expanded Uncertainty $k = 2$	$n^{2)}$	1507 mass fraction determined by event-specific rt-PCR ³⁾	
	[g/kg]	[g/kg]		[g/kg]	s
ERM-BF418a	< 0.5	-	5	< 0.4 ¹⁾	-
ERM-BF418b	1.0	-0.2; +0.6	5	1.0	0.2
ERM-BF418c	9.9	-0.6 ; +0.8	5	10.5 ⁴⁾	0.6
ERM-BF418d	98.6	-1.7 ; +2.0	5	101.9 ⁴⁾	4.5

¹⁾ The measured value was below the LOD of the method of 0.4 g/kg.

²⁾ For each mass fraction five randomly selected bottles were analysed. On each bottle five independent extracts were analysed in five replicates.

³⁾ Rt-PCR measures copy numbers of the targeted DNA sequence and was calibrated with known mass fractions of pure GM powder.

⁴⁾ Samples were diluted 5-times prior to measurements, in order to avoid inhibition of the PCR efficiency.

Results obtained with the event-specific rt-PCR method and being higher than the LOD (Table 10) are compared to the certified values in Figure 2. Quantification of the GM mass fraction of three mixtures of 1507 powders by rt-PCR proved to be consistent with the gravimetrically prepared mass fractions of CRM ERM-BF418. However, one has to be careful to draw quantitative conclusions from measurements of unknown samples as DNA and/or protein based GM quantification may vary with the particular maize variety tested.

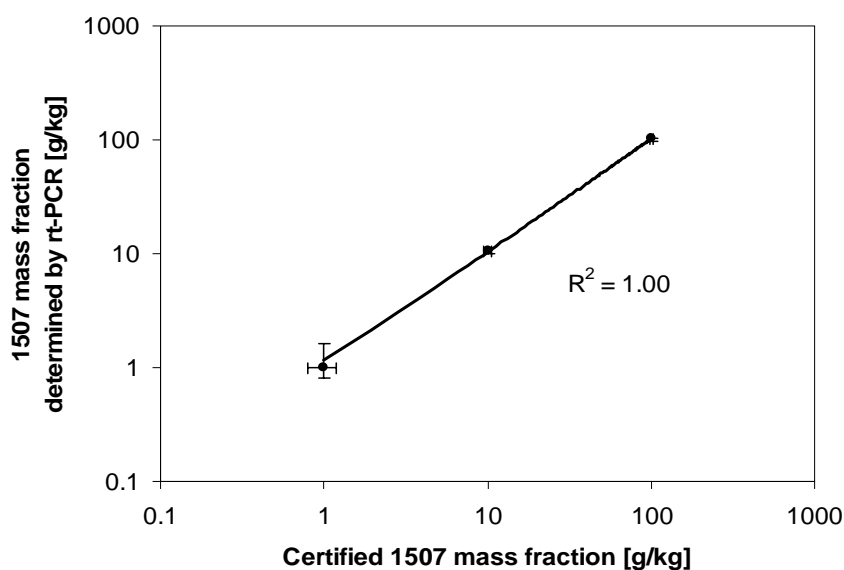


Figure 2: GM mass fractions of event 1507 quantified by event-specific rt-PCR versus certified 1507 mass fractions. Error bars of the certified mass fraction represent the expanded uncertainties; error bars of the mass fraction determined by rt-PCR represent s. The regression line is given.

References and acknowledgements

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Annex

Primer sequences used for the quantification of 1507 maize [4]

	Sequence (5' to 3')
MaiY-F1	TA _g TCT TC _g gCC AgA AT _g g
MaiY-R3	CTT TgC CAA gAT CAA gC _g
MaiY-S1	6-FAM- TAA CTC AA _g gCC CTC ACT CC _g -TAMRA
MaiJ-F2	TT _g gAC TA _g AAA TCT CgT gCT gA
mhmg-rev	gCT ACA TA _g ggA gCC TT _g TCC T
mhmg-Probe	6-FAM- CAA TCC ACA CAA AC _g CAC gC _g TA -TAMRA

Abstract

This report describes the preparation and certification of dry-mixed maize powder Certified Reference Materials (CRMs) with different mass fractions of genetically modified (GM) 1507 maize powder (CRMs ERM-BF418a, ERM-BF418b, ERM-BF418c and ERM-BF418d). The CRMs were processed in 2004 and certified in 2005 by the European Commission, Directorate General Joint Research Centre, the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium.

Seeds of non-modified maize and 1507 maize, both supplied by Pioneer Hi-Bred International (Johnston, IA, USA), were rinsed with demineralised water, drained and dried at 30 °C in order to minimise dust contamination from other crops. After a two step grinding process, transforming the seeds into a non-modified maize powder and a 1507 GM maize powder, the CRMs were gravimetrically prepared and homogenised by turbula- and dry-mixing.

The CRMs are intended for the quality control and calibration of methods for the detection and quantification of genetically modified food and feed. The 1507 mass fractions of ERM-BF418 were verified with the help of a DNA-based detection method. The CRMs are available in glass bottles containing 1 g of maize powder closed under argon atmosphere. The four CRMs belonging to the set ERM-BF418 were certified to contain the following 1507 mass fractions:

CRM	Certified value 1507 mass fraction ¹⁾ [g/kg]	Uncertainty ²⁾ [g/kg]
ERM-BF418a	< 0.5	not applicable
ERM-BF418b	1.0	-0.2; +0.6
ERM-BF418c	9.9	-0.6; +0.8
ERM-BF418d	98.6	-1.7; +2.0

¹⁾ The certified value is based on the mass fraction of dried non-genetically modified powder and dried genetically modified powder mixed and corrected for the water content. The certified value is traceable to the SI.

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 %.

The minimum sample intake recommended for analysis is 100 mg.

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