European Commission

European Reference Materials

CERTIFICATION REPORT

The certification of different mass fractions of DAS-40278-9 in maize seed powder

Certified Reference Materials ERM®-BF433a, ERM®-BF433b, ERM®-BF433c and ERM®-BF433d

EUR 25383 EN - 2012

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
Reference materials sales
Retieseweg 111
B-2440 Geel, Belgium
E-mail: jrc-irmm-rm-sales@ec.europa.eu
Tel.: +32 (0)14 571 705
Fax: +32 (0)14 590 406

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC 72351

EUR 25383 EN

ISBN 978-92-79-25315-7

ISSN 1831-9424

doi:10.2787/63375

Luxembourg: Publications Office of the European Union

Printed in Belgium

CERTIFICATION REPORT

The certification of different mass fractions of DAS-40278-9 in maize seed powder

Certified Reference Materials ERM®-BF433a, ERM®-BF433b, ERM®-BF433c and ERM®-BF433d

B. Dimitrievska, A.M. Kortekaas, P. Corbisier, M. Contreras, J. Seghers, S. Trapmann

European Commission, Joint Research Centre
Institute for Reference Materials and Measurements (IRMM)
Geel, Belgium

Summary

This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF433a, b, c and d, matrix materials certified for their DAS-40278-9 mass fractions. The material has been produced following ISO Guide 34:2009 [1].

Genetically modified (GM) seeds of the maize event DAS-40278-9 and of a non-GM maize variety were ground to obtain GM and non-GM base powders. Gravimetric mixtures of non-GM and GM maize powder were prepared by dry-mixing.

Between unit-heterogeneity has been quantified and stability during dispatch and storage have been assessed in accordance with ISO Guide 35:2006 [2].

The certified value was obtained from the gravimetric preparations, taking into account the purity of the base materials and their respective 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 17025:2005 [3]).

Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible heterogeneity, instability, and characterisation.

The materials are intended for the calibration or quality control of DAS-40278-9 maize identification and quantification methods. As any reference material, they can also be used for control charts or validation studies. The CRMs are available in glass vials containing at least 1 g of dried maize seed powder, closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.

The CRM has been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The following values were assigned:

	DAS-40278-9 Mass Fraction [1]	
	Certified value [2] [g/kg]	Uncertainty [3] [g/kg]
ERM-BF433a	< 0.3	-
ERM-BF433b	5.0	0.6
ERM-BF433c	10.0	0.9
ERM-BF433d	100	8

1) Genetically modified maize DAS-40278-9 with the unique identifier DAS-4Ø278-9.

2) Mass fraction of DAS-40278-9 maize based on the masses of genetically modified DAS-40278-9 maize seed powder and non-modified maize seed powder and their respective water content. The certified values and their uncertainties are traceable to the International System of Units (SI).

3) The certified uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Table of contents

Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression y = a + bx
CRM	Certified reference material
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EC	European Commission
ERM®	Trademark of European Reference Materials
EU	European Union
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurements *[ISO/IEC Guide 98-3:2008]*
EDTA	Ethylenediamintetraacetic acid
IEC	International Electrotechnical Commission
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
LOD	Limit of detection
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
n.c.	Not calculated
PCR	Polymerase Chain Reaction
PSA	Particle size analysis
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
RSE	Relative standard error (=RSD/$\sqrt{n}$)
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation; an additional index "rel" is added as appropriate
t	Time
t_i	Time point for each replicate
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
TE	Tris-EDTA
u	Standard uncertainty
U	Expanded uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-unit heterogeneity that could be hidden by the intermediate precision of the method; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit heterogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate

u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
VIM	Vocabulaire International de Métrologie – Concepts Fondamentaux et Généraux et Termes Associés (International Vocabulary of Metrology – Basic and General Concepts and Associated Terms) *[ISO/IEC Guide 99:2007]*
V-KFT	Volumetric Karl Fischer titration
$\overline{x}$	Arithmetic mean
$\overline{\overline{y}}$	Mean of all results of the homogeneity study
ν	Degrees of freedom

1 Introduction

1.1 Background: need for the CRM

Legislation in the European Union regulates the placing on the market of food and feed consisting of, containing or produced from genetically modified organisms (GMOs). They are referred to as genetically modified (GM) food and feed and require authorisation before being placed on the market in the European Union. Food and feed material which contains, consists of or is produced from GMOs in a proportion higher than 0.9 percent of the food and feed ingredient considered individually or food or feed consisting of a single ingredient, need to be labelled [5]. In general, this threshold demands on the one hand the development and validation of reliable methods for GMO quantification, and on the other hand the production of reference materials for calibration or quality control of these methods.

Dow AgroSciences (Oxon, UK) has developed the genetically modified (GM) maize event DAS-40278-9 with the unique identifier code DAS-4Ø278-9 following Commission Regulation (EC) No 65/2004 [6]. In 2010 the Institute for Reference Materials and Measurements (IRMM, Geel, BE) was asked to produce a reference material for the quantification of DAS-40278-9 maize. The event is designed to confer tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) and certain aryloxyphenoxypropionate (AOPP) herbicides (e.g. quizalofop). The herbicide tolerance in DAS-40278-9 maize was achieved through expression of the *aad-1* gene isolated from *Sphingobium herbicidovorans* that encodes the synthesis of aryloxyalkanoate dioxygenase-1 (AAD-1) protein [7]. The Certified Reference Material (CRM) produced by IRMM has been named ERM-BF433 and is composed of a set of four CRMs with different mass fractions of DAS-40278-9 maize.

1.2 Choice of the material

The set of CRMs ERM-BF433 was produced from ground GM seeds and non-GM seeds. Seeds were selected as a source for the raw material due to their high purity, compared to other materials.

1.3 Design of the project

Besides the non-GM pure material, all the gravimetric mixtures of non-GM and GM maize powder were prepared by dry-mixing. The first material ERM-BF433d was prepared by mixing pure non-GM with GM maize powder. ERM-BF433c was prepared by further dilution of ERM-BF433d and ERM-BF433b was prepared by further dilution of ERM-BF433c, in both cases with non-GM maize powder.

The different mass fractions of ERM-BF433 were certified using a gravimetric approach and details are described in this certification report.

2 Participants

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM and to ISO/IEC 17025 for the quantification of GMOs, BELAC No. 268-TEST)

3 Material processing and process control

3.1 Origin of the starting material

For the preparation of the CRMs, Dow AgroSciences supplied non-GM maize seeds and DAS-40278-9 maize seeds to IRMM. After arrival, the maize seeds were stored at (4 ± 3) °C in the dark until used for processing.

The purities of the delivered non-GM and GM maize seeds were investigated at IRMM using different approaches. For the GM maize seeds, a number of randomly selected seeds were analysed individually by real-time PCR to confirm the presence of the DAS-40278-9 event. Statistics were applied to calculate the purity of the GM seed batch. The purity of the non-GM maize seeds has been investigated after milling of the seeds by applying real-time PCR on the powder material.

The purity of the delivered GM maize seed batch had been measured by Dow AgroSciences testing 300 individual seeds with a lateral flow test strip. All seeds tested positive. The purity was measured at IRMM by analysing 200 randomly selected GM seeds for the presence of the GM event DAS-40278-9. The seeds were planted and genomic DNA was extracted from the seedling's leaves using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). Quantitative real-time PCR was then performed according to the event-specific real-time PCR method delivered under confidentiality agreement to IRMM. This method will be published after completion of its international validation on the homepage of the European Reference Laboratory for GM food and feed (EURL-GMFF) [8]. Genomic DNA extracted from pure DAS-40278-9 maize powder was used as positive control. Detection was done on an ABI 7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [9]. The results showed that all plants from the GM seed batch gave a signal for presence of the DAS-40278-9 event. Statistical analysis of the 200 measurements (Poisson distribution for rare events) revealed that the GM maize seed batch had a genetic purity of > 98.5 % (95 % level of confidence). The calculated lot purity of the GM seed batch was taken into account for the estimation of the uncertainties associated to the certified values of the reference materials (Section 6.2).

The purity of the non-GM seed batch was investigated on the processed powder. Real-time PCR measurements on the non-GM maize seed powder were performed with a limit of detection (LOD) for the mass fraction of 0.3 g/kg. The method did not detect the event DAS-40278-9 (Section 3.5). The LOD of the method was taken into account for calculating the certified value (Section 7.1).

3.2 Processing

All maize seeds received by IRMM were rinsed with water, drained, and dried on special trays in a drying chamber of a freeze-dryer at 30 °C for 20 hours (Epsilon 2-65D, Osterode, DE). The mass fraction of water was determined by volumetric Karl Fischer titration (V-KFT). After the washing and drying step, the non-GM seeds had a remaining residual water mass fraction of about 55 g/kg and the GM seeds had a remaining residual water mass fraction of about 44 g/kg.

About 30 kg of non-GM maize seeds and 15 kg of DAS-40278-9 maize seeds were used for the processing of ERM-BF433. The GM and non-GM base materials were processed separately. Cross-contamination and contamination with foreign DNA were avoided applying systematic cleaning, clean laboratory clothing and measures to prevent cross-contamination

by air. All contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to exposure to the 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 seeds were milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). Prior to this milling step, the maize seeds were frozen overnight in approximately 6 kg portions in stainless steel containers immersed in liquid nitrogen. The mill was also cooled to process the seeds at a temperature below -90 °C. The feeding speed of the mill was adjusted to ensure most efficient milling with respect to the particle size obtained. After milling, the powder was kept at (4 ± 3)°C. The GM and non-GM powders were then sieved separately with a 500 µm stainless steel mesh on a sieving machine (Russel Finex, London, UK). In case of the GM powder, a coarse fraction of 26 g did not pass the 500 µm mesh and was discarded. For the non-GM powder a coarse fraction of 58 g did not pass the 500 µm mesh and was discarded. The remaining powder of each base material, which passed the sieve, was mixed in a DynaMIX CM200 (WAB, Basel, CH) for one hour to improve equal distribution of the different types of maize tissues because the milling and sieving processes applied foster the separation of the different tissues from each other.

For the non-GM and GM powders, a residual water mass fraction of (105.5 ± 19.8) g/kg and (115.2 ± 21.7) g/kg respectively was measured by Volumetric Karl Fischer titration (V-KFT, 758 KFD Titrino, Metrohm, Herisau, CH) with the expanded uncertainty calculated with $k = 2$. In order to facilitate the dry mixing, the water content was reduced. The powders were dried overnight under vacuum in a freeze-dryer (Epsilon 2-65D, Osterode, DE) at 30 °C. The final water mass fractions ($N = 1$, $n = 5$) of the non-GM powder and the GM powder were (17.2 ± 2.2) g/kg (U, $k = 2$) and (16.0 ± 2.0) g/kg (U, $k = 2$) respectively. The particle volumes for both powders were measured based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and compared (Figure 1). The mean particle diameters ($N = 1$, $n = 5$), calculated by the PSA software, were 66 µm ($s = 4$ µm) for the non-GM powder and 69 µm ($s = 4$ µm) for the GM powder. It is important to understand that the cumulative volume distribution of particles derived from laser light scattering data 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 calculated volume of the particles based on their diameter is, therefore, overestimating the mean particle size. It has been concluded that the particle volume fractions of the non-GM and GM base powders were sufficiently similar to allow the processing of mixtures without introducing a bias based on the DNA extractability.

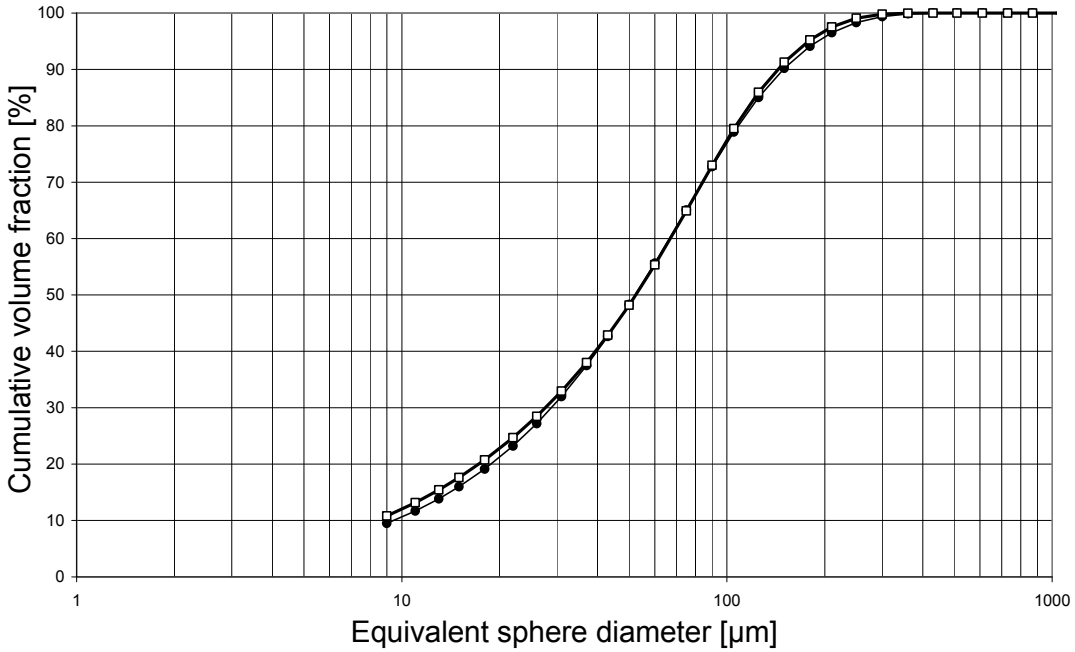

Figure 1: Accumulation of particle volume fractions in the GM powder (•) and non-GM powder (□) analysed by PSA. Each point represents the mean of five replicates (*N* = 1, *n* = 5). The total volume is set as 100 %.

The ground base materials were used to produce a blank material for DAS-40278-9 (non-GM seed powder) and three mixtures containing different mass fractions of DAS-40278-9 maize seed powder in non-GM maize seed powder at nominal mass fraction levels of 5, 10 and 100 g/kg. The term "nominal" is used to discriminate between the value targeted in the processing and the certified value assigned after completion of the certification process.

All these materials, including the blank powder, were treated according to the same procedure. The powder materials were weighed using a calibrated balance with an intermediate precision, expressed as relative standard uncertainty, of 0.1 %. Calibration of the balance is carried out on an annual basis by an external company accredited for ISO/IEC 17025 calibration services; additionally the performance of the balance is verified before use. Portions were weighed into a container and mixed for 30 min by DynaMIX and further homogenised in a propeller mixer for additional 2 min. The blank material was processed first, followed by the mixtures. For the preparation of the mixtures, the masses of the non-GM and GM powders were corrected for their respective water mass fractions. The masses which are theoretically needed to reach a certain nominal mass fraction were calculated. During the certification process, dry masses have been used to establish the certified mass fraction (Section 7.1). The material having a nominal mass fraction of 100 g DAS-40278-9/kg was produced by mixing pure GM with pure non-GM ground base materials. The material having a nominal mass fraction of 10 g/kg DAS-40278-9 was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder and the material with a nominal mass fraction of 5 g/kg was thereafter produced by further dilution of the 10 g/kg GM powder with pure non-GM powder. At each mixing step, the water mass fraction of the mixed materials was taken into account (Table 4). The gravimetric preparation formed the basis for the calculation of the mass fraction of the powders (Section 6).

After finalisation of the mixing steps, the powders were filled 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 necks. Before final closure of the vials, air was evacuated in a freeze-dryer and replaced by argon. The vials were finally closed inside the freeze-dryer 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 mass fraction levels of DAS-40278-9: nominal 0 g/kg = silver (BF433a), nominal 5 g/kg = blue (BF433b), nominal 10 g/kg = red (BF433c), nominal 100 g/kg = brown (BF433d), consistent with the cap colours of previous IRMM CRMs for GMOs. Each of the vials was identified by a numbered label indicating the ERM code and the unit number. Following the inventory 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 ± 3) °C.

3.3 Process control

The residual mass fraction of water in ten randomly selected bottles from each of the powder materials was determined by V-KFT. The results are summarised in Table 1.

Table 1: Water mass fraction in candidate CRMs ERM-BF433 determined by V-KFT (*N* = 10, *n* = 1)

Candidate CRM	Water mass fraction [g/kg]	
	$\overline{x}$	***U (k = 2)***
ERM-BF433a	12.9	1.2
ERM-BF433b	14.2	1.4
ERM-BF433c	13.7	1.3
ERM-BF433d	21.3	2.0

Five randomly selected bottles from each of the powder materials were analysed twice for their particle volume distribution (*N* = 5, *n* = 2) based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). The powders have a particle diameter below 610 µm (Figure 2). The mean particle diameters (*N* = 5, *n* = 2), calculated by the PSA software, were 73 µm (*s* = 6 µm), 70 µm (*s* = 3 µm), 69 µm (*s* = 3 µm) and 59 µm (*s* = 7 µm) for ERM-BF433a, b, c and d, respectively.

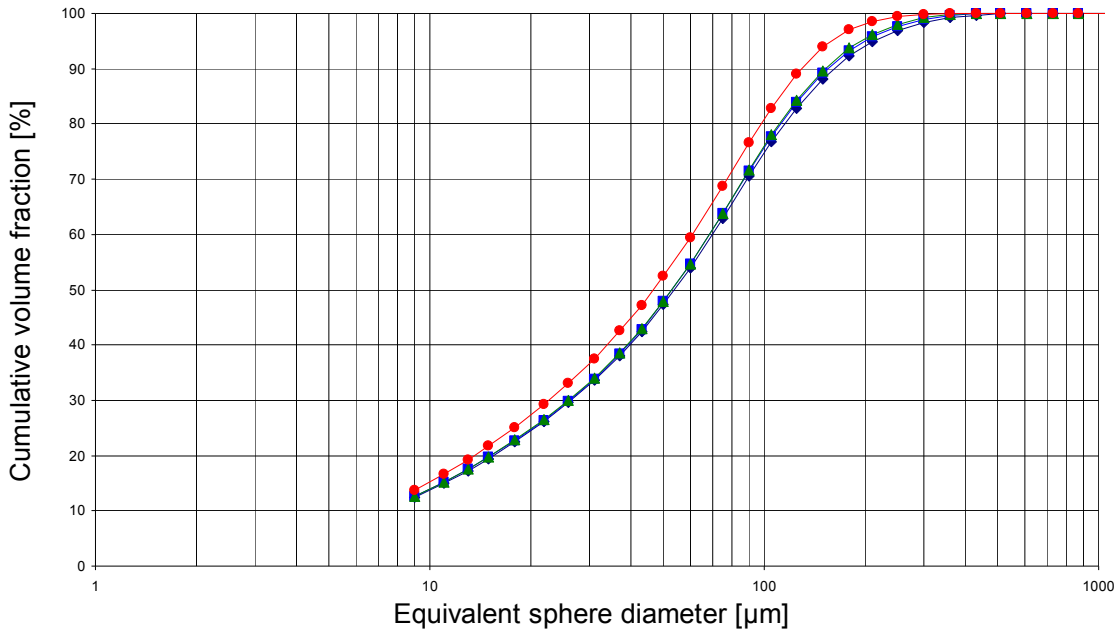

Figure 2: Accumulation of particle volume fractions in ERM-BF433a (◆), ERM-BF433b (■), ERM-BF433c (▲) and ERM-BF433d (●) analysed by PSA (*N* = 5, *n* = 2). Each point represents the mean of two measurements (*N* = 1, *n* = 2). The total volume was set as 100 %.

3.4 DNA content of the base materials

Three of the described CRMs are mixtures of GM and non-GM maize seed powders, produced gravimetrically and intended to be used for calibration or quality control of quantitative measurements of the genomic DNA, following DNA extraction and purification. Any DNA mass fraction difference in the non-GM and GM base materials will lead to a shift of the measurement results obtained with e.g. real-time PCR.

The mass of DNA in both base materials was estimated using a slight modification of the classical fractionation method developed initially by Ogur and Rosen [10]. A sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds and acidic extraction with 0.84 mol/L perchloric acid pH 0.3 at 70 °C was carried out. The mass of DNA was determined after derivatisation with diphenylamine using a spectrophotomer. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [10, 11]. The extractable DNA mass fraction of the two materials was calculated as:

$$\frac{\textit{DNA mass extracted from 100 mg GM maize powder}}{\textit{DNA mass extracted from 100 mg non-GM maize powder}}$$

The ratio of the DNA mass extractable from 100 mg of GM and non-GM maize powder was found to be 0.98 ± 0.06 (*N* = 9 with an expanded uncertainty, *k* = 2). A *t*-test showed with 95 % confidence that no significant difference exists between the DNA mass extracted from the GM and non-GM maize powders using the modified Ogur and Rosen method.

The DNA integrity was checked by gel electrophoresis. From 200 mg samples of the processed powder materials ERM-BF433a, BF433b, BF433c and BF433d, DNA was extracted using a CTAB DNA extraction method (Annex A). Approximately 1.0 µg DNA were loaded on an agarose gel (mass concentration of 7.5 agarose g/L). Staining of the DNA was done with an ethidium bromide solution (0.5 mg/L). None of the samples showed DNA degradation (data not shown).

3.5 Confirmation measurements

As a control for the gravimetric preparations, the mass fraction of DAS-40278-9 maize in all four CRMs was confirmed by the confidential real-time PCR method provided by Dow AgroSciences targeting the transgenic DNA insertion in this maize and using a sample intake of 10 g. The real-time PCR test was calibrated with genomic DNA extracted from the pure DAS-40278-9 maize powder. At IRMM, the genomic DNA was extracted by in-house validated CTAB extraction method (Annex A) using 200 mg powder samples. After the extraction, it was diluted in a TE buffer solution (pH 8.0, 1 mmol/L TRIS and 0.01 mmol/L EDTA) and used to produce calibration curves for the target taxon-specific gene and the transgene as well. For the calibration curve of the target taxon-specific gene the DNA was used undiluted and diluted up to 200-times. For the calibration curve of the transgene the DNA was diluted between 4-times and 2000-times. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of DAS-40278-9 and the obtained Ct-values. The diluted DNA was used to establish the calibration points for the transgene. 3.3-times *s* of the lowest calibration point at which RSD was below 25 % was taken to calculate the LOD of the PCR method. The results of the quantification of DAS-40278-9 are shown in Table 2. Quantification of the mass fraction of DAS-40278-9 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF433. However, as no independent calibration was carried out, the data displayed in Table 2 can be used for confirmation of the processing, but do not necessarily resemble the true value. It has to be noted that the calibrant used for the transgenic and the taxon-specific target is genomic DNA extracted from the pure DAS-40278-9 maize powder.

Table 2: Quantification of the DAS-40278-9 maize mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure DAS-40278-9 seed powder for calibration

Candidate CRM	DAS-40278-9 maize mass fraction [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF433a	< 0.3 [1) 2)]	-
ERM-BF433b	5.0 [3)]	0.6
ERM-BF433c	10.2 [3)]	1.2
ERM-BF433d	102.5 [1)]	11.8

[1)] Mean for 3 samples (extraction replicates) from each of 5 random selected bottles (N = 5, n = 3), with each sample measured in three real-time PCR replicates.

[2)] The obtained value is below the LOD determined during method validation (0.3 g/kg).

[3)] Mean values of 12 bottles were included in the calculation. Five of them were measured in triplicates (N = 5, n = 3), while seven were done in duplicates (N = 7, n = 2), with each sample measured in three real-time PCR replicates.

4 Homogeneity

A key requirement for any reference material is the equivalence between the various units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty associated with the certified value. In contrast to that, it is not relevant if the variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

This homogeneity study was planned together with the measurements to control the gravimetric preparations and the short-term stability study (Sections 3.5 and 5.1). As the measurement results were obtained under intermediate precision conditions on bottles randomly taken from the entire batch and analysed in a randomised order, they were as well suited to investigate the homogeneity. Due to the high intermediate precision of the in-house validated method, two replicates of each sample were analysed in this study. Homogeneity of the blank material is demonstrated by the test for the purity of the non-GM base material (Section 6.1). No specific homogeneity study was done for ERM-BF433a.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRMs are valid for all bottles of the material, within the stated uncertainties.

For the between-unit homogeneity test, the number of selected bottles corresponds to approximately the cubic root of the total number of the produced bottles. Therefore, 12 bottles were selected for ERM-BF433b and ERM-BF433c. In order to facilitate homogeneity studies and short-term stability study, 15 bottles were selected for ERM-BF433d. For each CRM a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12 and 15 groups respectively (with similar number of bottles) and one bottle was randomly selected from each group. From each bottle, 2 independent samples were taken and analysed by real-time PCR. Due to the number of PCR plates required the measurements were performed under intermediate precision conditions. Samples were analysed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. The results are shown in the figures in Annex B.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. A trend in the analytical sequence was found for ERM-BF433b (95 % confidence level), pointing to a drift of the signal in the analytical system. The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value. Correction of non-significant trends is therefore expected to improve the sensitivity of subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95% confidence level were corrected as shown below [12].

$$corrected\,result = measured\,result - b \cdot i$$

b = slope of the linear regression

i = position of the result in the analytical sequence

The trend-corrected dataset was tested for consistency using Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. Analyses of the individual results and unit means have detected one outlier. The outlying data came from not

sufficiently randomised samples that were analysed with all three replicates on a single real-time PCR plate.

Regression analyses were performed to evaluate potential trends and have shown no trends present in the analytical sequence and in the filling sequence of ERM-BF433d as well.

Quantification of between-unit heterogeneity is most easily done by analysis of variance (ANOVA), which can separate the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the repeatability of the method if the individual samples are representative for the whole bottle.

Evaluation by ANOVA requires bottle means which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. Distribution of the bottle means was tested using histograms and normal probability plots. Too few data are available for each mean to make a clear statement of the distribution of individual results. Therefore, it was checked whether all individual data follow a unimodal distribution using histograms and normal probability plots.

One has to bear in mind that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum heterogeneity that could be hidden by the intermediate precision of the method, was calculated as described by Linsinger *et al.* [13]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum heterogeneity that might be undetected by the given study setup.

For this certification project the intermediate precision of the method ($s_{wb,rel}$), between–unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}\sqrt[4]{\frac{2}{v_{MSwithin}}}}}{\bar{y}}$$

MS_{within}	mean square within a unit from an ANOVA
$MS_{between}$	mean squares between-unit from an ANOVA
$\bar{y}$	mean of all results of the homogeneity study
n	mean number of replicates per unit
$v_{MSwithin}$	degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 3.

Table 3: Between-unit variation established within the homogeneity studies

CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF433b	10.0	5.4	3.8
ERM-BF433c	10.9	n.c.[1)]	4.1
ERM-BF433d	7.9	n.c.[1)]	3.4

[1)] n.c. - cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying means measured per CRM unit and no significant trends (95 % confidence level) in the filling sequence. Therefore the between-unit standard deviation can be used as estimate of u_{bb}. As u^*_{bb} sets the limits for the detection power of the study, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential heterogeneity.

4.2 Within-unit homogeneity and minimum sample intake

Homogeneity and stability experiments were performed using a 200 mg sample intake. Using this sample intake the acceptable intermediate precision was achieved, demonstrating that the within-unit heterogeneity does no longer contribute to analytical variation at this level.

5 Stability

Time, temperature and radiation were regarded as the most relevant influences on stability of the materials. The influence of ultraviolet or visible radiation was minimised by the choice of the containment which eliminates most of the incoming light. In addition, materials are stored and dispatched in the dark, thus practically eliminating the possibility of radiative degradation. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C could be reached and stability against these conditions must be demonstrated if transport at ambient temperature will be applied.

The short-term stability study has been carried out using an isochronous design [14]. In that approach, samples of ERM-BF433d were stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible ("reference conditions"), effectively "freezing" the degradation status of the materials. At the end of the isochronous storage, the samples were analysed simultaneously under intermediate precision conditions. Analysis of the material (after various exposure times and temperatures) under intermediate precision conditions greatly improves the sensitivity of the stability tests.

ERM-BF433 is a dried maize powder which has been processed similarly to other GMO CRM maize powders. As the water content of these powders and their particle size are similar, stability data obtained in the frame of the stability monitoring of maize GMO CRMs were used for the estimation of the uncertainty instead of an individual long-term stability study.

5.1 Short-term stability study

For the short-term stability study, samples of ERM-BF433d have been stored at 4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Five units per storage time were selected using a random stratified sampling scheme. Five units that were kept at the reference temperature were measured in three extraction replicates from each unit. The rest of the units that were kept at different temperatures (4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks at each temperature) were analysed in two extraction replicates, measured by real-time PCR. The measurements were performed under intermediate precision conditions with respect to the PCR plates, and in a randomised manner to be able to separate a potential analytical drift from a trend over storage time.

The obtained data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test. No outliers were detected.

Furthermore, the data were plotted against storage time and regression lines of mass fraction versus time were calculated. No outliers were observed. The slopes of the regression lines were not significantly different from 0 (99 % confidence level) for 4°C, 18 °C and 60 °C.

The material therefore can be dispatched without further precautions under ambient conditions.

The results of the measurements are shown in Annex C.

5.2 Long-term stability study

Data from the stability-monitoring program for GMO CRMs were available. The GMO content in the maize powder CRMs (ERM-BF411d, ERM-BF412d, ERM-BF413d, ERM-BF413ek, ERM-BF414d, ERM-BF415d, ERM-BF416c, ERM-BF417c, ERM-BF418c, ERM-BF420b, ERM-BF423b, ERM-BF424c, ERM-BF427b and ERM-BF427c) has been measured at 61 occasions over a period of 7 years. At each occasion, measurements were performed simultaneously on one PCR plate on units stored at +4 °C and -70 °C under intermediate precision conditions. In fact, each of these studies can be seen as a two-point isochronous study. The evaluation is based on the ratio of samples from +4 °C and -70 °C.

The results were screened for outliers using the single and double Grubbs test. One outlier was detected (ERM-BF411d). This outlier was retained for the further statistical analyses because the individual values for storage at 4 °C and storage at reference temperature were in agreement with the estimated uncertainty.

To verify that the data obtained from stability monitoring can be used to estimate the stability uncertainty contribution for ERM-BF433, an additional isochronous study was organised within the frame of the short-term stability assessment (Section 5.1). The data of the 4 °C short-term stability study did not contradict with the data obtained from the stability monitoring.

Based on these measurements, it can be concluded that the dried maize material can be stored at 4 °C.

The results of the measurements are shown in Annex D.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can rule out degradation of materials completely, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method intermediate precision, i.e. to estimate the uncertainty of stability. This means, even under ideal conditions, the outcome of a stability study can only be "degradation is (0 ± x) % per time".

Uncertainties of stability during dispatch and storage were estimated as described in [15]. For this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contributions u_{sts} and u_{lts} are then calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as

$$u_{sts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{tt}$$

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{sl}$$

RSD	relative standard deviation of all results of the stability study
x_i	result at time point *i*
$\bar{x}$	mean results for all time points
t_{tt}	chosen transport time (1 week at 60 ºC)
t_{sl}	chosen shelf life (24 months at 4 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. The uncertainty describes the possible change during a dispatch at 60 °C lasting for one week and was estimated to be 0.6%.
- $u_{lts,rel}$, the stability during storage. The uncertainty contribution describes the possible degradation during 24 months storage at 4 °C and was estimated to be 1.3%.

The uncertainties of the short-term and long-term stability have been considered in the combined uncertainty (Section 7). After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to control its further stability.

6 Characterisation by gravimetric preparation

The material characterisation was based on a primary method of measurement, confirmed by an independent method. A primary method of measurement (also called "primary reference method" in the International Vocabulary of Metrology (VIM) [16]) is a method that does not require calibration with a standard of the same measurand and does not depend on a chemical reaction. Such methods are of highest metrological order and often yield results with very low uncertainties. However, it is nevertheless prudent to demonstrate absence of bias or gross errors by use of an independent method of lower metrological order.

For ERM-BF433 gravimetric mixing was chosen as the method of choice. The four candidate CRMs under the label code ERM-BF433 are maize powder materials processed from non-GM and GM seeds. While ERM-BF433a is prepared from the pure blank material, the other CRMs of the series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF433 is being certified for the mass fraction of DAS-40278-9 maize.

6.1 Purity of the base materials

The purity of the GM and non-GM batches used for the processing of these powders was investigated in order to be able to calculate the certified value. No indication was found that the GM DAS-40278-9 maize material contained seeds being negative for the event DAS-40278-9 (Section 3.1). No indication for the presence of DAS-40278-9 was found in the non-GM powder by real-time PCR (Section 3.5). As no evidence for a contamination was found in both base materials, 100 % purity was used for the calculation of the certified mass fraction of DAS-40278-9 in the powder mixtures. The difference between the statistically established purity of 98.5 % (Section 3.1) and the 100 % purity is taken into account in the uncertainty calculation.

The non-GM powder used for the production of ERM-BF433 did not contain traces of the DAS-40278-9 above the LOD of the applied real-time PCR method (Section 3.5). The certified value for ERM-BF433a is therefore based on the LOD of the real-time PCR method applied, as determined during in-house method validation.

6.2 Mass fractions and their uncertainties

The certified mass values are based on the mass fractions of dry-mixed GM and non-GM powders, corrected for their water mass fractions, and taking into account the powders' purity with regard to the DAS-40278-9 event. The values were calculated according to the following equations:

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

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

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

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

In Table 4, the data supporting the calculation of the mass fractions of DAS-40278-9 maize are summarised.

Table 4: Subsequent mixing of GM DAS-40278-9 maize seed powder with non-GM powder (ERM-BF433a) to prepare the ERM-BF433b, c and d materials

CRM	GM powder			Non-GM powder [1)]	Mixtures
	Mass fraction of GM powder [g/kg]	Water mass fraction ± *U* (*k* = 2) [g/kg]	Mass [g]	Mass [g]	Resulting mass fraction of GM powder [g/kg]
ERM-BF433d	1000.0	16.0 ± 2.0	399.0	3600.4	100.0
ERM-BF433c	100.0 [2)]	15.1 ± 1.9	399.2	3600.8	10.0
ERM-BF433b	10.0 [3)]	14.9 ± 1.9	1498.6	1501.8	5.0

[1)] The non-GM powder (ERM-BF433a) used for the gravimetric preparations had a water mass fraction of 17.2 ± 2.2 g/kg (*U, k* = 2) and was considered to be free of DAS-40278-9 maize.

[2)] For the preparation of ERM-BF433c the 100 g/kg GM powder (ERM-BF433d) was used.

[3)] For the preparation of ERM-BF433b the 10 g/kg GM powder (ERM-BF433c) was used.

The uncertainties on the certified mass fractions *(*u_{char}) of DAS-40278-9 maize is 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 $u_{char,4}$). 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 98.5 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value (Table 5).

Table 5: Uncertainty budgets for the mass fractions of DAS-40278-9 maize in ERM-BF433

CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]				Combined uncertainty u_{char} [g/kg]
		$u_{char,1}$ [1)]	$u_{char,2}$ [2)]	$u_{char,3}$ [3)]	$u_{char,4}$ [4)]	
ERM-BF433a	0	n.a	n.a	0.0953	n.a	0.0953
ERM-BF433b	5	0.0128	0.0107	0.0953	0.0216	0.0991
ERM-BF433c	10	0.0243	0.0186	0.0953	0.0432	0.1090
ERM-BF433d	100	0.1721	0.1518	0.0953	0.4317	0.4982

[1)] Standard uncertainty of the mass determination mainly based on the uncertainty of the balance and the number of weighing steps required.

[2)] Standard uncertainty of the water mass fraction determination by V-KFT.

[3)] Standard uncertainty of the purity estimation of the non-GM base material (LOD = 0.3 g/kg), based on the half-width of the interval between 0 and 0.3 g/kg, divided by the square root of 3 (rectangular distribution).

[4)] Standard uncertainty of the purity estimation of the GM starting material (> 98.5 %), based on the interval between 98.5 % and 100 % divided by the square root of 3 (rectangular distribution).

6.3 Verification measurements

Gel electrophoresis proved that the DNA analyte was not degraded during processing of the CRM (Section 3.4). Real-time PCR measurements demonstrated that no mixing errors were made (Section 3.5).

7 Value Assignment

For these materials certified values have been assigned and full uncertainty budgets in accordance with the Guide to the Expression of Uncertainty in Measurement [4] were established.

The certified values are based on the masses of dried powders 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 during the preparation of the materials (Table 4).

The assigned uncertainty is calculated from uncertainties related to characterisation, u_{char} (Section 6.2), potential between-unit heterogeneity, u_{bb} (Section 4.1) and potential degradation during transport (u_{sts}) and long-term storage, u_{lts} (Section 5). These different contributions were combined to estimate the expanded uncertainty of the certified value (U_{CRM}) with a coverage factor k as

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

- u_{char} was estimated as described in Section 6.2
- u_{bb} was estimated as described in Section 4.1
- u_{sts} was estimated as described in Section 5.1 and 5.3
- u_{lts} was estimated as described in Section 5.2 and 5.3

For the blank material, the LOD of the method was used to describe the 95 % confidence interval on the certified mass fraction of the event (< 0.3 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 U = 0.2 g/kg, which is below the certified < 0.3 g/kg value. The LOD is, therefore, already a conservative estimate of the certified value and no uncertainty is assigned.

A coverage factor k of 2 was applied to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 6.

Table 6: Certified values and their uncertainties for ERM-BF433

CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{lts} [g/kg]	U_{CRM} [g/kg] [2)]
BF433a	< 0.3 [1)]	0.0953	n.a	n.a	n.a	-
BF433b	5.0	0.0991	0.2697	0.0300	0.0649	0.6
BF433c	10.0	0.1090	0.4095	0.0599	0.1298	0.9
BF433d	100	0.4982	3.3957	0.5992	1.2984	8

[1)] With a 95 % confidence the certified value is below this level.
[2)] Expanded (k = 2) and rounded uncertainty.

As it is not known how the certified GM powder mass fractions are related to the corresponding transgenic and target taxon-specific DNA copy number ratio, the user is reminded that IRMM only certifies these materials for their mass fraction of DAS-40278-9. Additionally, 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 quantification of GMOs may vary with the particular matrix and the variety tested.

8 Metrological traceability and commutability

8.1 Metrological traceability

Quantity value

The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure. The value is therefore traceable to the SI.

8.2 Commutability

Many measurement procedures include one or more steps, which are selecting specific (or specific groups of) analytes from the sample for the subsequent steps of the whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all the analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is nowadays summarised in a concept called 'commutability of a reference material'. There are various definitions expressing this concept. For instance, the CSLI Guideline C-53A [17] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and, thus, is a crucial characteristic in case of the application of different measurement methods. When commutability of a CRM is not established in such cases, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as calibrant.

The CRM is prepared from non-GM and GM maize seed powder and the analytical behaviour will be the same as for a routine sample of ground maize seeds/grains. For other types of samples the commutability has to be assessed.

9 Instructions for use

9.1 Storage conditions

The materials shall be stored at +4 °C in dark. The materials are hygroscopic and therefore the user is reminded to close bottles immediately after taking a sample.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples.

9.2 Safety and protection for the environment

The usual laboratory safety measures apply. The material is for in-vitro use only; it does not contain any viable seeds.

9.3 Minimum sample intake

The minimum sample intake is 200 mg.

9.4 Use of the certified value

The main purpose of these materials is the use for calibration or quality control of DAS-40278-9 maize detection methods. As any reference material, they can also be used for control charts or validation studies.

Use as a calibrant

If this matrix material is used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty. Furthermore, it should be noted that using the same material for calibration and quality control limits the control possibilities as calibrant and quality control material are based on the same raw materials. If unavoidable, it is recommended to use different concentration levels of ERM-BF433 for calibration and for quality control.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [18]).

For assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_m).
- Combine measurement uncertainty (u_m) with the uncertainty of the certified value (u_{CRM}): $u_\Delta = \sqrt{u_m^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_Δ) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_m \leq U_\Delta$ then there is no significant difference between the measurement result and the certified value, at a confidence level of about 95 %.

Use in quality control charts

The materials can be used for quality control charts. Different units of the same CRM code will give the same result as heterogeneity was included in the uncertainties of the certified values.

Acknowledgments

The authors would like to acknowledge the support received from J. Charoud-Got, P. Conneely, H. Emteborg and M. Thumba from IRMM related to the processing of this CRM.

Furthermore, the authors would like to thank A. Lamberty (IRMM) and J. Simonet (IRMM) for the reviewing of the certification report, as well as the experts of the Certification Advisory Panel "Biological Macromolecules and Biological/Biochemical Parameters", A. Heissenberger (Umweltbundesamt GmbH, Vienna, AT), M. Wagner (University for Veterinary Medicine Vienna, AT) and L. Siekmann (University of Bonn, DE) for their constructive comments.

References

[1] ISO Guide 34:2009, General requirements for the competence of reference materials producers, International Organization for Standardization, Geneva, Switzerland

[2] ISO Guide 35:2006, Reference materials – General and statistical principles for certification, International Organization for Standardization, Geneva, Switzerland

[3] ISO/IEC 17025:2005, General requirements for the competence of testing and calibration laboratories, International Organization for Standardization, Geneva, Switzerland

[4] ISO/IEC Guide 98-3:2008, Guide to the Expression of Uncertainty in Measurement, International Organization for Standardization, Geneva, Switzerland

[5] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. Off. J. Eur. Union, L 268:1-28

[6] 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

[7] EFSA (2011) Application for authorisation of DAS-40278-9 maize grain for all uses as for any other maize, excluding cultivation, according to Articles 5 and 17 of Regulation (EC) No 1829/2003 on genetically modified food and feed, Part II, http://registerofquestions.efsa.europa.eu/roqFrontend

[8] European Reference Laboratory for GM food and feed (EURL GMFF) Status of dossiers http://gmo-crl.jrc.ec.europa.eu/statusofdoss.htm

[9] TaqMan® Universal PCR Master Mix protocol, Applied Biosystems. See website http://docs.appliedbiosystems.com/pebiodocs/04304449.pdf

[10] Ogur M., Rosen G. (1950) The Nucleic Acids of Plant Tissues. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. 25:262-276

[11] Ganguli P.K. (1970) A sensitive procedure for the estimation of deoxyribonucleic acid by the diphenylamine reaction in the presence of cupric sulfate. Rev. Can. Biol. 29: 339-346.

[12] G. O'Donnell, D. Brynn Hibbert (2005) Treatment of bias in estimating measurement uncertainty, Analyst 130:721-729

[13] T.P.J. Linsinger, J. Pauwels , A.M.H. van der Veen, H. Schimmel, A. Lamberty (2001) Homogeneity and stability of reference materials, Accred. Qual. Assur. 6: 20-25

[14] A. Lamberty, H. Schimmel, J. Pauwels, (1998) The study of the stability of reference materials by isochronous measurements, Fres. J. Anal. Chem. 360: 359-361

[15] T.P.J. Linsinger, J. Pauwels, A. Lamberty, H. Schimmel, A.M.H. van der Veen, L. Siekmann (2001) Estimating the Uncertainty of Stability for Matrix CRMs, Fres. J. Anal. Chem. 370: 183-188

[16] ISO Guide 99, International vocabulary of metrology - Basic and general concepts and associated terms (VIM), International Organization for Standardization, Geneva, Switzerland, 2007

[17] H. Vesper, H. Emons, M. Gnezda, C. P. Jain, W. G. Miller, R. Rej, G. Schumann, J. Tate, L. Thienpont, J. E. Vaks (2010) Characterization and Qualification of Commutable Reference Materials for Laboratory Medicine; Approved Guideline, CLSI document C53-A, Clinical and Laboratory Standards Institute, Wayne, PA, USA

[18] T.P.J. Linsinger, ERM Application Note 1: Comparison of a measurement result with the certified value, www.erm-crm.org

Annexes

Annex A:

CTAB DNA extraction method (in-house modified)

Chemicals

CTAB buffer A
2 % (m/v) CTAB
1.4 M NaCl
0.1 M Tris-HCl
15 mM Na_2EDTA

CTAB buffer B
1 % (m/v) CTAB
0.1 M Tris-HCl
15 mM Na_2EDTA

TE buffer
Tris 1 mM, pH 8.0
0.01 mM EDTA

Chloroform : Octanol (24:1) (u/v)
RNase A (100 mg/ml)
Proteinase K (20 mg/ml)
Ethanol abs.
Ethanol 70%

a) Weigh 200 mg powder in 2 mL microcentrifuge tube.
b) Add 1 mL of preheated (65 °C) CTAB buffer A and mix thoroughly by shaking or tapping the tube.
c) Add 10 µL of RNase A (100 mg/mL) and mix shortly by shaking.
d) Incubate minimum 15 min at 65 °C (optionally mix a few times by shaking).
e) Add 20 µL of Proteinase K (20 mg/mL) and mix shortly by shaking.
f) Incubate minimum 15 min at 65 °C (optionally mix a few times by shaking).
g) Spin down cell debris 10 min at 16000x*g*.
h) Transfer supernatant to a 1.5 mL microcentrifuge tube containing 500 µL of Chloroform:Octanol (24:1).
i) Vortex or shake 10 s, then centrifuge 10 min at 16000x*g*.
j) Transfer upper phase to a new 1.5 mL microcentrifuge tube containing roughly an equal volume of Chloroform:Octanol (24:1) (this can be estimated from the volume transferred in step h).
k) Vortex or shake 10 s, then centrifuge 5 min at 16000x*g*.
l) Transfer upper phase to a 2 mL tube, carefully determining the volume transferred (if unsure, pipet up and down once more).
m) Add 2 volumes of CTAB buffer B and mix by pipetting up and down.
n) Incubate 1 h at room temperature to precipitate the DNA.
o) Centrifuge 10 min at 16000x*g*; carefully decant the supernatant and discard it.

p) Resuspend the precipitate in 400 µL of 1.2 M NaCl; vortex gently.

q) Add 400 µL of Chloroform:Octanol (24:1), vortex/shake 10 s, then centrifuge 5 min at 16000x*g*.

r) Transfer the upper phase to a 1.5 mL tube, carefully determining the volume transferred (if unsure, pipet up and down once more).

s) Add 2 volumes of cold (-20 °C) absolute ethanol, mix gently by inverting the tube (if DNA strands are not visible at this phase, additionally incubate the samples for 30 min at -20 °C).

t) Centrifuge minimum 10 min at 16000x*g* at 4 °C.

u) Carefully decant the supernatant and wash the pellet by addition of 500 µL cold (-20 °C) 70 % ethanol.

v) Vortex shortly, then centrifuge 5 min 16000x*g* at 4 °C.

w) Remove the supernatant using a 1 mL pipette or by decanting it (optionally, spin again shortly and remove the remaining liquid with a low volume pipette).

x) Allow the pellet to air-dry few minutes (or use vacuum drying).

y) Resuspend the DNA pellet in 100 µL of TE buffer preheated at 50 °C.

Annex B: Results of the homogeneity measurements

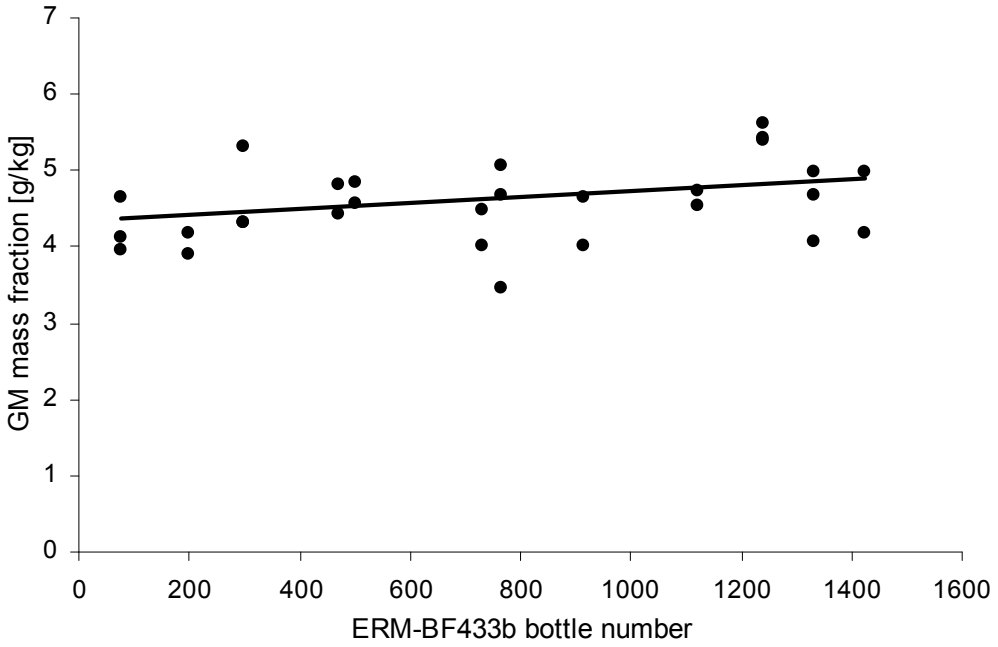

Figure B1: Real-time PCR measurement results obtained for ERM-BF433b (*N* = 12). Five of the samples were measured three times (*N* = 5, *n* = 3), while seven were measured twice (*N* = 7, *n* = 2). All measurements were carried out in triplicate on real-time PCR plate. The linear regression for all data points is given.

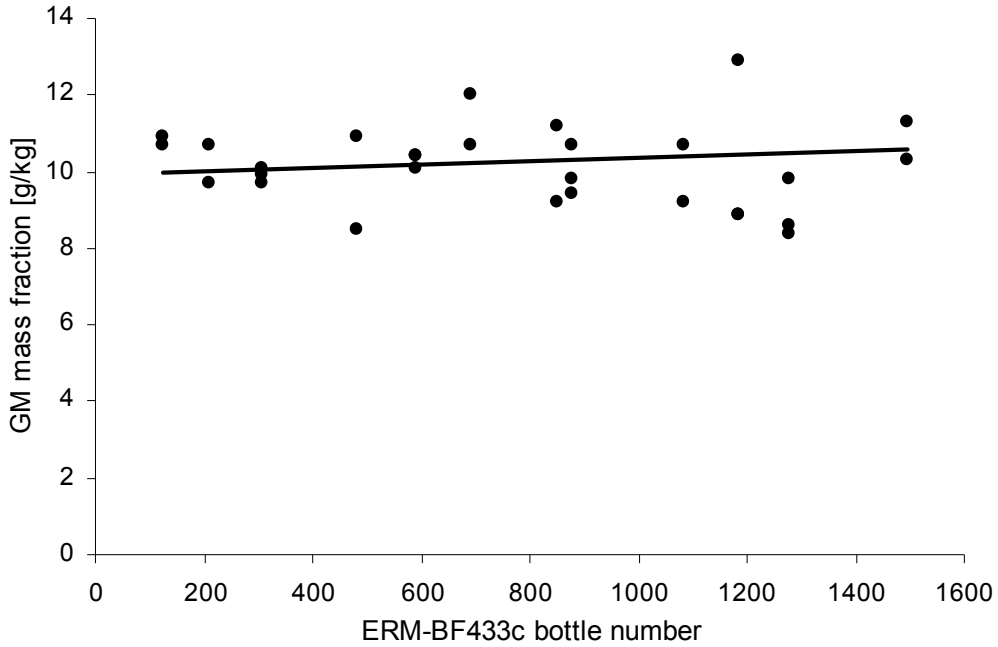

Figure B2: Real-time PCR measurement results obtained for ERM-BF433c (*N* = 12). Five of the samples were measured three times (*N* = 5, *n* = 3), while seven were measured twice (*N* = 7, *n* = 2). All measurements were carried out in triplicate on real-time PCR plate. The linear regression for all data points is given.

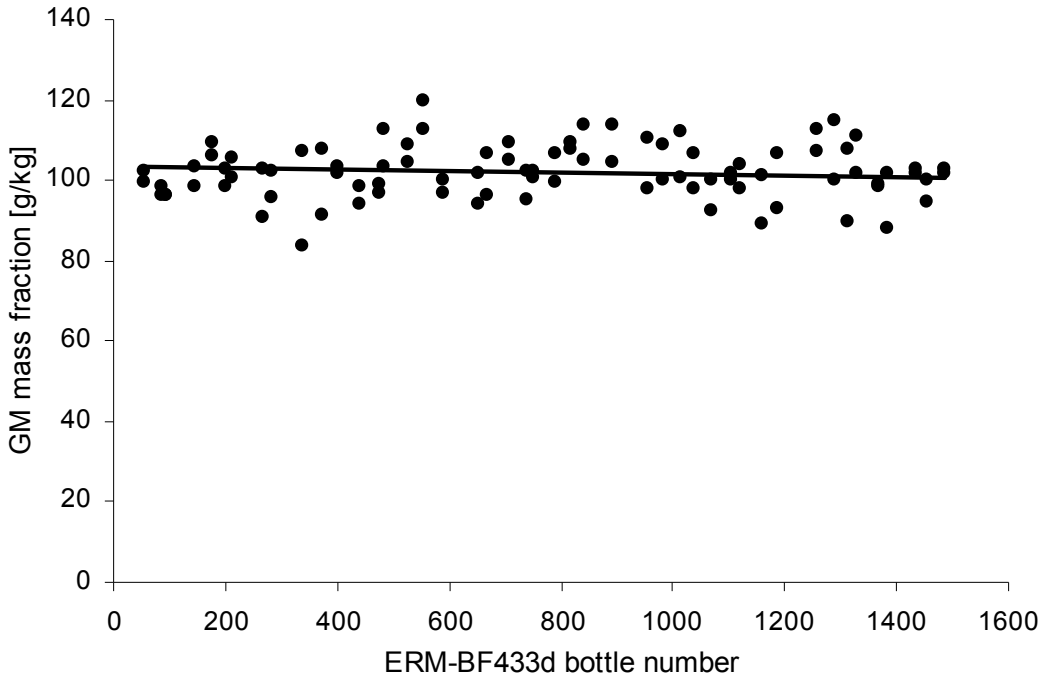

Figure B3: Real-time PCR measurement results obtained for ERM-BF433d (*N* = 45, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

Annex C: Results of the short-term stability measurements

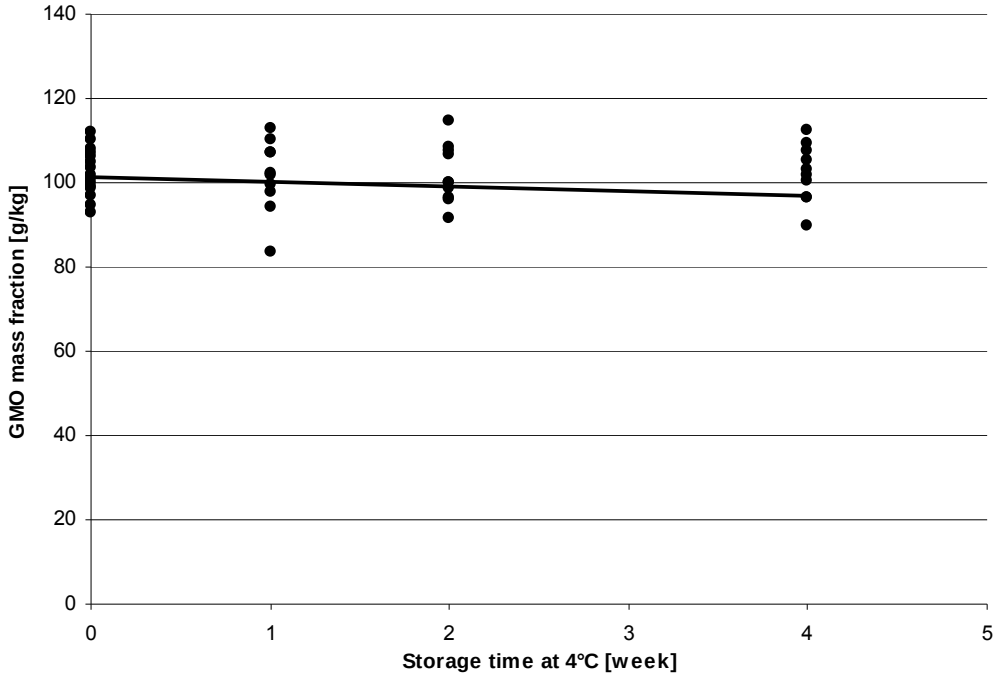

Figure C1: Real-time PCR measurement results obtained for ERM-BF433d during short-term stability testing at 4°C (*N* = 5, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

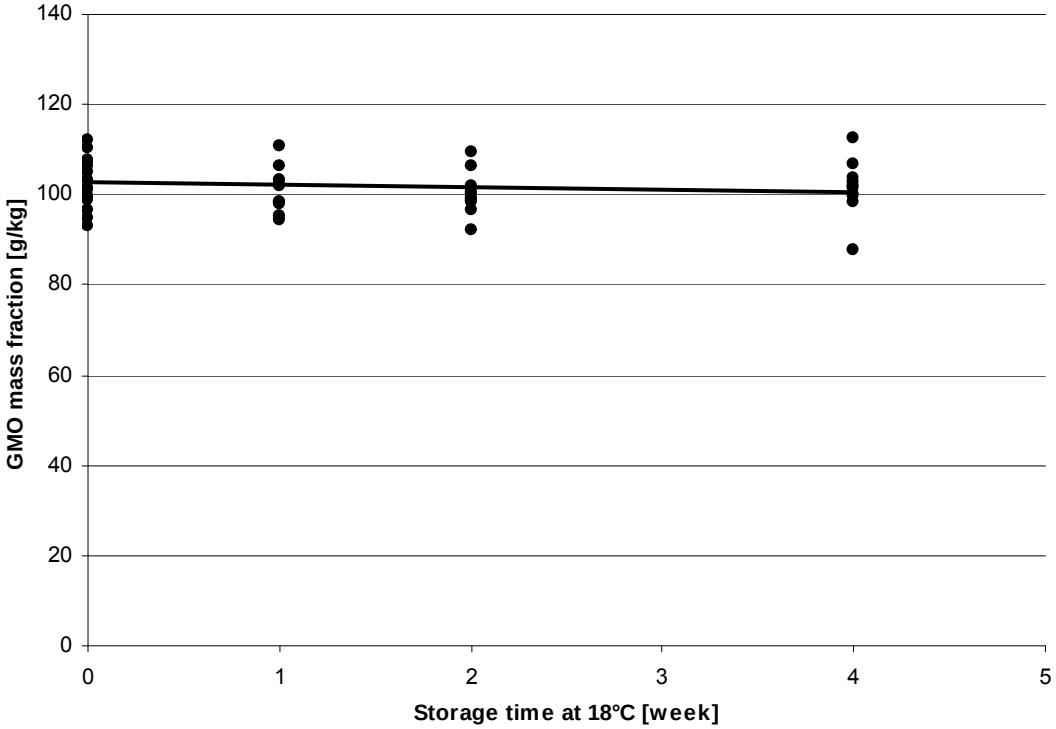

Figure C2: Real-time PCR measurement results obtained for ERM-BF433d during short-term stability testing at 18°C (*N* = 5, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

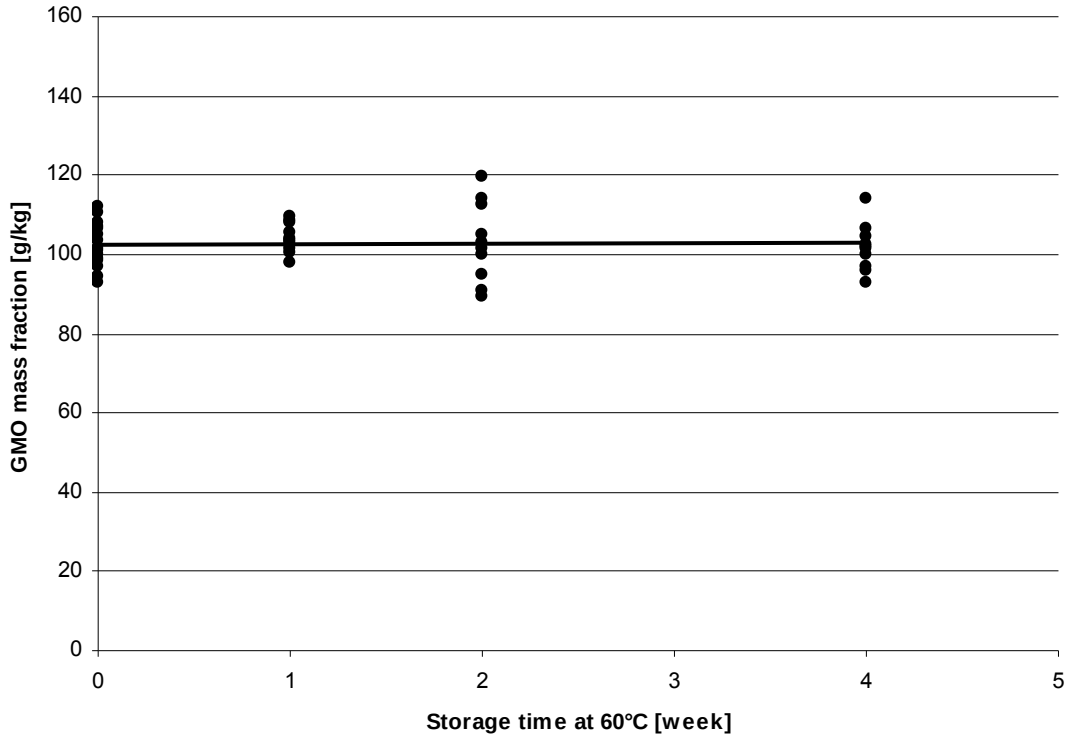

Figure C3: Real-time PCR measurement results obtained for ERM-BF433d during short-term stability testing at 60°C (*N* = 5, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

Annex D: Results of the long-term stability measurements

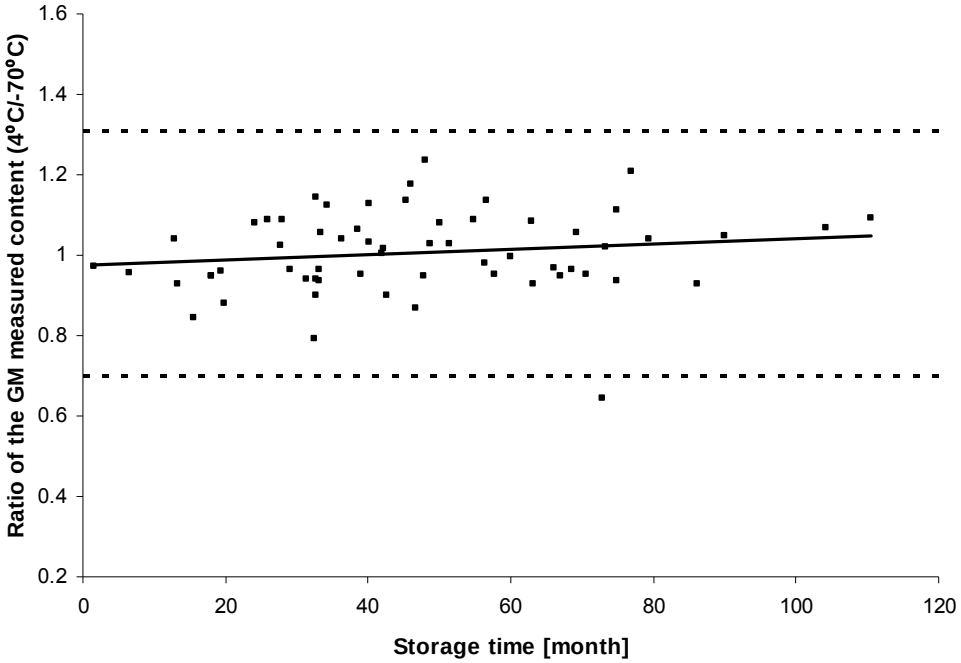

Figure D1: Real-time PCR measurement results obtained for ERM-BF411d, ERM-BF412d, ERM-BF413d, ERM-BF413ek, ERM-BF414d, ERM-BF415d, ERM-BF416c, ERM-BF417c, ERM-BF418c, ERM-BF420b, ERM-BF423b, ERM-BF424c, ERM-BF427b and ERM-BF427c during post certification monitoring. The dashed lines give the limits of 3*s* obtained for the measurement results. The linear regression for all data points is given.

European Commission

EUR 25383 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: **The certification of different mass fractions of DAS-40278-9 in maize seed powder - Certified Reference Materials ERM®-BF433a, ERM®-BF433b, ERM®-BF433c and ERM®-BF433d**
Author(s): B. Dimitrievska, A.M. Kortekaas, P. Corbisier, M. Contreras, J. Seghers, S. Trapmann
Luxembourg: Publications Office of the European Union
2012 – 33 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1831-9424
ISBN 978-92-79-25315-7
doi:10.2787/63375

Abstract
This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF433a, b, c and d, matrix materials certified for their DAS-40278-9 mass fractions. The material has been produced following ISO Guide 34:2009 [1]. Genetically modified (GM) seeds of the maize event DAS-40278-9 and of a non-GM maize variety were ground to obtain GM and non-GM base powders. Gravimetric mixtures of non-GM and GM maize powder were prepared by dry-mixing. Between unit-heterogeneity has been quantified and stability during dispatch and storage have been assessed in accordance with ISO Guide 35:2006 [2]. The certified value was obtained from the gravimetric preparations, taking into account the purity of the base materials and their respective 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 17025:2005 [3]). Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible heterogeneity, instability, and characterisation. The materials are intended for the calibration or quality control of DAS-40278-9 maize identification and quantification methods. As any reference material, they can also be used for control charts or validation studies. The CRMs are available in glass vials containing at least 1 g of dried maize seed powder, closed under argon atmosphere. The minimum amount of sample to be used is 200 mg. The CRM has been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.
The following values were assigned:

	DAS-40278-9 Mass Fraction [1]	
	Certified value [2] [g/kg]	Uncertainty [3] [g/kg]
ERM-BF433a	< 0.3	-
ERM-BF433b	5.0	0.6
ERM-BF433c	10.0	0.9
ERM-BF433d	100	8

1) Genetically modified maize DAS-40278-9 with the unique identifier DAS-4Ø278-9.
2) Mass fraction of DAS-40278-9 maize based on the masses of genetically modified DAS-40278-9 maize seed powder and non-modified maize seed powder and their respective water content. The certified values and their uncertainties are traceable to the International System of Units (SI).
3) The certified uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Key policy areas include: environment and climate change; energy and transport; agriculture and food security; health and consumer protection; information society and digital agenda; safety and security, including nuclear; all supported through a cross-cutting and multi-disciplinary approach.

LA-NA-25383-EN-N

ISBN 978-92-79-25315-7