European Commission

ERM
European Reference Materials

CERTIFICATION REPORT

The certification of different mass fractions of DP-ØØ4114-3 in maize seed powder

Certified Reference Materials ERM®-BF439a, ERM®-BF439b, ERM®-BF439c, ERM®-BF439d and ERM®-BF439e

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Abstract
This report describes the production of a set of Certified Reference Materials (CRMs) ERM BF439a, b, c, d and e, which are certified for their 4114 maize event mass fractions. These materials were produced following ISO Guide 34:2009 and are certified in accordance with ISO Guide 35:2006. Genetically modified (GM) seeds of the 4114 maize event and seeds from a non-GM maize variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM maize seed powder were prepared gravimetrically.
The certified value was obtained from the gravimetric preparations, taking into account the genetic purity with respect to the event 4114 maize of the two powder materials and their water mass fractions. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements were within the scope of accreditation to ISO/IEC 17025:2005).
The uncertainties of the certified values were estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties relating to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).
The materials are intended for the calibration or quality control of real-time PCR measurements to identify 4114 maize and/or quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs are available in glass bottles containing at least 1 g of dried maize seed powder, which were sealed under an atmosphere of argon. The minimum amount of sample to be used for extraction of the DNA is 200 mg.
The CRMs were accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

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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.

Summary

This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF439a, b, c, d and e, which are certified for their 4114 maize event mass fractions. These materials were produced following ISO Guide 34:2009 [1] and are certified in accordance with ISO Guide 35:2006 [2].

Genetically modified (GM) seeds of the 4114 maize event and seeds from a non-GM maize variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM maize seed powder were prepared gravimetrically.

The certified value was obtained from the gravimetric preparations, taking into account the genetic purity with respect to the event 4114 maize of the two powder materials and their water mass fractions. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements were within the scope of accreditation to ISO/IEC 17025:2005 [3]).

The uncertainties of the certified values were estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties relating to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).

The materials are intended for the calibration or quality control of real-time PCR measurements to identify 4114 maize and/or quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs are available in glass bottles containing at least 1 g of dried maize seed powder, which were sealed under an atmosphere of argon. The minimum amount of sample to be used for extraction of the DNA is 200 mg.

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

The following values were assigned:

	4114 maize mass fraction [1]	
	Certified value [g/kg]	Uncertainty [g/kg] [5]
ERM-BF439a	< 0.06 [2]	-
ERM-BF439b	> 986 [3]	-
ERM-BF439c	1.00 [4]	0.13
ERM-BF439d	10.0 [4]	1.1
ERM-BF439e	100 [4]	8

1) Genetically modified maize with the unique identifier DP-ØØ4114-3-maize.

2) The certified reference material has been produced from conventional, non-modified maize seeds. No contamination was detected in this material when using an event-specific real-time polymerase chain reaction assay targeting the 4114 maize event. The limit of detection (LOD) was 0.06 g/kg. With 95 % confidence, the true 4114 maize mass fraction of the material is below 0.06 g/kg. The certified value is traceable to the International System of units (SI).

3) This certified reference material was produced from genetically modified 4114 maize seeds. The certified value is based on the genetic purity of the maize powder with regard to 4114 maize. In total 207 seeds were tested individually for the presence of the 4114 maize event by event-specific real-time polymerase chain reaction. All seeds tested positive. With 95 % confidence, the true 4114 maize mass fraction of the material is above 986 g/kg. The certified value is traceable to the International System of units (SI).

4) This certified value is based on the masses of dried genetically modified 4114 maize powder and dried non-modified maize powder that were mixed, taking into account their respective genetic purity with regard to 4114 maize and their respective water content. The certified value is traceable to the International System of units (SI).

5) The 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.

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Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression y = a + *b*x
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
Cry	Crystal toxin (protein)
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
g	Relative centrifugal force
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurements *[ISO/IEC Guide 98-3:2008]*
EDTA	Ethylenediaminetetraacetic acid
IEC	International Electrotechnical Commission
IHCP	Institute for Health and Consumer Protection
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
JRC	Joint Research Centre
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
rpm	Revolutions per minute
RT	Room temperature
s	Sample standard deviation
$s_{\bar{x}}$	Standard deviation of the estimate of the mean (also referred to as standard error of the estimate of the mean)
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
SI	The International System of Units
s_{rel}	Relative standard deviation (also referred to as RSD)
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 inhomogeneity 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 inhomogeneity; 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
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

The European Union has legislation which regulates placing on the market any food or feed which consists of, contains, or is produced from genetically modified organisms (GMOs). These items are referred to as genetically modified food and feed and require authorisation for marketing in the European Union. They will additionally need to be labelled if they contain more than 0.9 % of GMOs [5]. This labelling threshold is applicable for the adventitious presence of GMOs, whilst GMOs that are intentionally added need to be labelled independently from any threshold. However, feed may contain 0.1 (m/m) % of a GMO for which an authorisation process is pending, or for which authorisation in the EU has expired [6]. These thresholds require the development and validation of reliable methods for GMO quantification, and the production of reference materials for calibration or quality control of these methods.

DuPont Pioneer (US) developed the genetically modified (GM) 4114 maize event (unique identifier code DP-ØØ4114-3, following Commission Regulation (EC) No 65/2004 [7]) as a transgenic insect resistant and herbicide tolerant crop. The 4114 maize event was developed by *Agrobacterium*-mediated transformation with plasmid PHP27118. The event contains 4 cassettes (cry1F, cry34Ab1, cry35Ab1 and pat gene), which confer resistance to certain lepidopteran and coleopteran pests, as well as tolerance to the herbicide phosphinothricin [8]. In 2014 DuPont Pioneer commissioned the Institute for Reference Materials and Measurements (IRMM, Geel, BE) to produce a certified reference material (CRM) for the quantification of 4114 maize. The CRM produced by IRMM has been named ERM-BF439 and is composed of a set of five CRMs containing different mass fractions of 4114 maize.

1.2 Choice of the material

The set of CRMs ERM-BF439 was produced from milled GM and non-GM seeds. Seeds (in contrast to the grains) were selected as the source of raw material because of their high degree of purity.

1.3 Design of the CRM project

The genetic purity with respect to the 4114 maize event of the non-GM and GM maize seeds was investigated.

Alongside the pure non-GM material ERM-BF439a and the pure GM material ERM-BF439b, mixtures of non-GM and GM maize powder were prepared gravimetrically. The first mixed material ERM-BF439e was prepared by mixing pure GM with non-GM maize powder. ERM-BF439d was prepared by further dilution of ERM-BF439e, and ERM-BF439c was prepared by further dilution of ERM-BF439d, in both cases with non-GM maize powder.

The different mass fractions of ERM-BF439 were certified using a gravimetric approach, the details of which are described in Section 6.

2 Participants

2.1 Provider of raw material and quantification method

Pioneer Overseas Corporation, Johnston, Iowa, US, provided the raw material on behalf of DuPont Pioneer.

Pioneer Hi-Bred International, Inc. Wilmington, Delaware, provided an event-specific real-time PCR method under a confidentially agreement with the IRMM.

2.2 Project management, processing, analytical measurements and evaluation

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 GMO quantification, BELAC No. 268-TEST)

3 Material processing and process control

3.1 Origin of the starting material

Pioneer Overseas Corporation, Johnston, Iowa, US, supplied the IRMM with non-GM maize seeds and 4114 maize seeds to prepare candidate CRMs. According to the information provided by DuPont Pioneer, the 4114 maize seeds were from hemizygous plants selected through successive breeding generations. The donor for the 4114 maize event was the female parent. After arrival, the seeds were stored at (4 ± 3) °C in the dark until processing.

The genetic purity with respect to the 4114 maize event of the GM maize seeds was assessed at IRMM by analysing 207 randomly selected seeds for the presence of the GM 4114 maize event. Genomic DNA was extracted from plants grown from individual seeds using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). The event-specific real-time PCR method to detect the 4114 maize event received from Pioneer was first validated in-house and was afterwards applied to verify the presence of the 4114 maize event in the plants grown from the received seeds. Once this method has been internationally validated by the European Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF), it will be published on the EURL homepage [9]. Genomic DNA extracted from pure 4114 maize powder was used as positive control using the CTAB method (Annex A). Amplification and detection was performed on an ABI 7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, US) [10]. All DNA extracts tested positive for the presence of the 4114 maize event. Statistical analysis of the 207 measurements (Poisson distribution for rare events) indicated that the GM maize seed batch had a genetic purity > 98.6 % (95 % level of confidence). This was taken into consideration during the estimation of the uncertainties associated with the certified values of the CRMs (Section 6.2).

The genetic purity of the non-GM seed batch with respect to the 4114 maize event was investigated using the processed seed powder. Five bottles of ERM-BF439a were randomly selected and the DNA was extracted from two samples taken from each bottle (extraction replicates, $N = 5$, $n = 2$). Each DNA extract was then analysed in 3 replicates by real-time PCR method, with a limit of detection (LOD) of 0.06 g/kg. This analysis did not detect the 4114 maize event (Section 3.4). The LOD of the event-specific real-time PCR method was taken into consideration when the certified value of ERM-BF439a was calculated (Section 7).

3.2 Processing and process control

All maize seeds received by the IRMM were rinsed with water, drained, and dried on trays in the drying chamber of a freeze-dryer at 25 °C for 20 h (Epsilon 2-100D, Martin Christ, Osterode, DE).

Approximately 30 kg of non-GM maize seeds and 10 kg of 4114 maize event seeds were used for the production of the ERM-BF439.

The GM and non-GM base materials were processed separately. The risks of cross-contamination and contamination with foreign DNA were minimised by thorough cleaning, by wearing clean laboratory clothing and by using measures to prevent airborne cross-contamination. Additionally, all contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to contact with the base materials. An in-house validation study had previously shown that the solution degraded DNA effectively under the conditions applied. The maize seeds and the maize powders were stored for short periods of time in closed plastic containers at 4 °C.

The maize seeds were frozen overnight in liquid nitrogen in approximately 4 kg portions in stainless steel containers and were subsequently milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). The mill was maintained below -90 °C throughout the process. The feeding speed of the mill was optimised to ensure that the seeds were milled to the required particle size. After milling, the powder was maintained at (4 ± 3) °C. The GM and non-GM powders were then separately sieved with a 750 µm stainless steel mesh on a sieving machine equipped with an ultra-sound sieving aid (Russel Finex, London, UK). A coarse fraction of approximately 45 g and 94 g, for the GM and non-GM powders respectively, did not pass through the 750 µm mesh and were discarded. The remaining powder from each base material was mixed in a DynaMIX CM200 (WAB, Basel, CH) for 1 h to homogenize the distribution of the different types of maize seed tissues, since it is known that the milling and sieving processes encourage the separation of the various seed tissues from each other.

The residual water mass fractions of the non-GM and GM powders were measured by volumetric Karl Fischer titration (V-KFT) (841 KFD Tritino, Metrohm, Herisau, CH), as (91.4 ± 11.5) g/kg and (93.9 ± 11.8) g/kg respectively ($N = 1$, $n = 3$), with the expanded uncertainty calculated using a coverage factor $k = 2$. To facilitate gravimetric mixing, the water content of the powders was further reduced by drying overnight under vacuum, in the freeze-dryer at 25 °C. The final water mass fractions of the non-GM powder and the GM powder were (20.1 ± 2.5) g/kg (U, $k = 2$) and (18.9 ± 2.4) g/kg (U, $k = 2$), respectively ($N = 1$, $n = 3$) (Table 1).

The particle size distribution for both powders were measured based on deconvoluted laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and were compared (Figure 1). The cumulative volume distribution of the particles derived from laser scattering data is based on their equivalent spherical diameter, i.e. the diameter of the particles derived from the volume occupied upon their rotation. The mean particle diameter of the non-GM and GM base powders was 122.5 µm ± 11.8 µm (s) and 113.9 µm ± 7.3 µm (s), respectively. However, since most particles are not perfectly spherical, the calculated volume of the particles based on their diameter will therefore overestimate the mean particle size. Therefore a three point specification of the particle size distribution ($N = 1$, $n = 3$) was calculated, consisting of the equivalent sphere diameters where 10 %, 50 % and 90 % of the total volume distribution have a smaller particle size (Table 1). These size classes were denoted X_{10}, X_{50} and X_{90}, respectively. A t-test showed with 95 % confidence that there was no significant difference between the X_{10}, X_{50}, X_{90} values and between the mean particle diameter of the non-GM and GM maize powders. Based on the particle volume distributions it was concluded that the non-GM and GM base powders were sufficiently similar and they could be processed further without introducing a bias which would subsequently affect the extractability of the DNA.

Table 1: The water mass fraction determined by V-KFT and additionally the particle diameter and particle size distribution based on deconvoluted laser diffraction patterns of the base materials

Base material	Water mass fraction [g/kg]		Mean particle diameter [µm]		Particle size distribution X_{10} [µm]		Particle size distribution X_{50} [µm]		Particle size distribution X_{90} [µm]	
	$\bar{x}$	U	$\bar{x}$	S	$\bar{x}$	U	$\bar{x}$	U	$\bar{x}$	U
Non-GM powder	20.1 [1)]	2.5	122.51 [2)]	11.79	15.91 [3)]	1.64	102.74 [3)]	6.7	258.63 [3)]	29.15
GM powder	18.9 [1)]	2.4	113.88 [2)]	7.31	14.32 [3)]	0.14	94.83 [3)]	3.34	241.49 [3)]	21.19

[1)] Mean of one sample (N = 1, n = 3). The associated expanded uncertainty (U) with a coverage factor k = 2 has been estimated during validation of the V-KFT method applied to maize powder.

[2)] Mean of one sample (N = 1, n = 3) with the sample standard deviation

[3)] Mean of one sample (N = 1, n = 3). Given are the equivalent sphere diameters where 10 %, 50 % or 90 % of the volume distribution have a smaller particle size. The associated expanded uncertainty (U) with a coverage factor of k = 2 has been estimated during validation of the particle size distribution measurement.

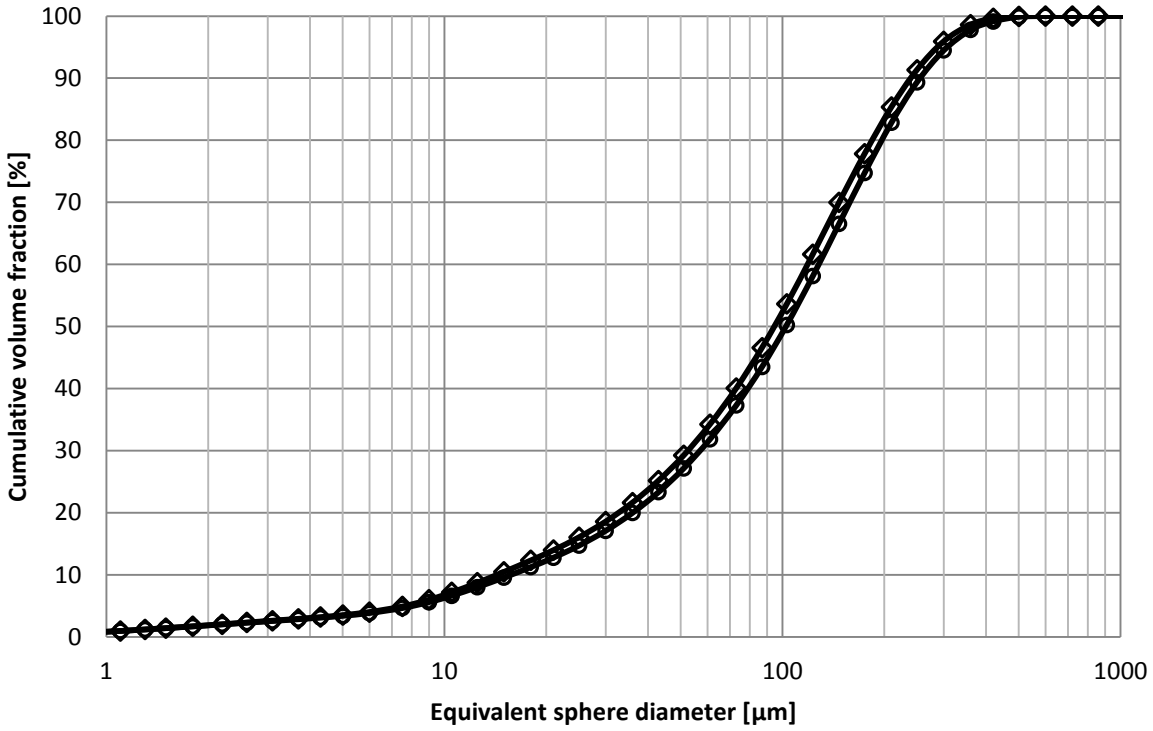

Figure 1: Volume-based cumulative distribution of equivalent sphere diameters in the GM powder (◇) and non-GM powder (○) analysed by laser diffraction (N = 1, n = 3). The total particle volume for each material is set as 100 %.

The milled base materials were used to prepare the blank material for 4114 maize (non-GM maize seed powder), the pure GM 4114 maize material and three mixtures at nominal mass fraction levels of 1, 10 and 100 g/kg 4114 maize event. The term "nominal" is used for the target value during the processing whereas the value assigned after completion of the certification process is called certified value.

All the materials produced were treated according to the same procedure and strict measures were taken to avoid cross-contamination. The powder materials were weighed using a calibrated balance (QC15DCE-S, Sartorius) with an intermediate precision, determined during calibration and expressed as standard uncertainty (u), of 0.15 g. Calibration of the balance is carried out on an annual basis by an external company (accredited under ISO/IEC 17025). The performance of the balance was verified before use on a daily basis by using in-house reference weights. The masses of the non-GM and GM powders, which are theoretically needed to reach a certain nominal mass fraction, were calculated. They were also corrected for their respective water content. Portions of the base materials were weighed into a container and mixed for 1 h by using a DynaMIX CM200. The material with a nominal 4114 maize mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM milled base materials. Similarly, the material with a nominal 4114 maize mass fraction of 10 g/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder and then the material with a nominal mass fraction of 1 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 materials was taken into account (Table 6). During the certification process, the gravimetric preparation was the basis for the calculation of the certified 4114 maize mass fraction for the three powder mixtures (Section 6).

The powders were filled into 10 mL brown glass bottles using an automatic filling device (All-Fill Sandy, UK). In order to avoid cross contamination the equipment was cleaned between each mass fraction level and the first 30 bottles of each batch were discarded as an additional precaution. The blank material was filled first, followed by the mixtures with increasing mass fraction with the pure GM material filled last. Lyophilisation inserts were automatically placed in the bottle necks. The bottles were then placed in a freeze-dryer (Epsilon 2-100D) to provide an argon atmosphere, and were closed inside the freeze-dryer with the help of a hydraulic device. Capping and labelling took place in a capping and labelling assembly from Bausch & Ströbel and BBK, respectively (Ilshofen and Beerfelden, both in Germany). Colour-coded caps were used to facilitate the identification of the different mass fraction levels of 4114 maize event: nominal 0 g/kg = silver (BF439a), nominal 1000 g/kg = black (BF439b), nominal 1 g/kg = gold (BF439c), nominal 10 g/kg = red (BF439d), nominal 100 g/kg = brown (BF439e), consistent with the cap colours of previous IRMM CRMs for GMOs. Each of the bottles was identified by a numbered label indicating the ERM code and the unit number according to filling order. After the inventory and the selection of bottles for future analysis according to a random stratified sampling scheme, the remaining bottles were stored in the dark at 4 °C (+6 °C / -3 °C).

Five randomly selected bottles from each of the powder materials were measured by V-KFT to determine the residual mass fraction of water in the candidate CRMs. The results are summarised in Table 2.

Table 2: Water mass fraction of candidate ERM-BF439 CRMs determined by V-KFT (N = 1, n = 5). The associated expanded uncertainty (U) has been estimated during validation of the V-KFT method on maize powder

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	U (k = 2)
ERM-BF439a	19.3	2.9
ERM-BF439b	19.1	2.8
ERM-BF439c	18.5	2.7
ERM-BF439d	17.3	2.6
ERM-BF439e	17.7	2.6

The particle size distribution in the candidate CRMs was determined based on the deconvoluted laser diffraction pattern of the constituent powders. Five randomly selected bottles from each of the candidate CRM were analysed twice (N = 5, n = 2). The diameter of all particles was below 500 µm (Figure 2). The mean particle diameters and standard deviations of the mean, measured by laser diffraction, were 131 µm ($s_{\bar{x}}$ = 8 µm), 106 µm ($s_{\bar{x}}$ = 21 µm), 131 µm ($s_{\bar{x}}$ = 10 µm), 126 µm ($s_{\bar{x}}$ = 20 µm) and 128 µm ($s_{\bar{x}}$ = 4 µm) for ERM-BF439a, b, c, d and e, respectively.

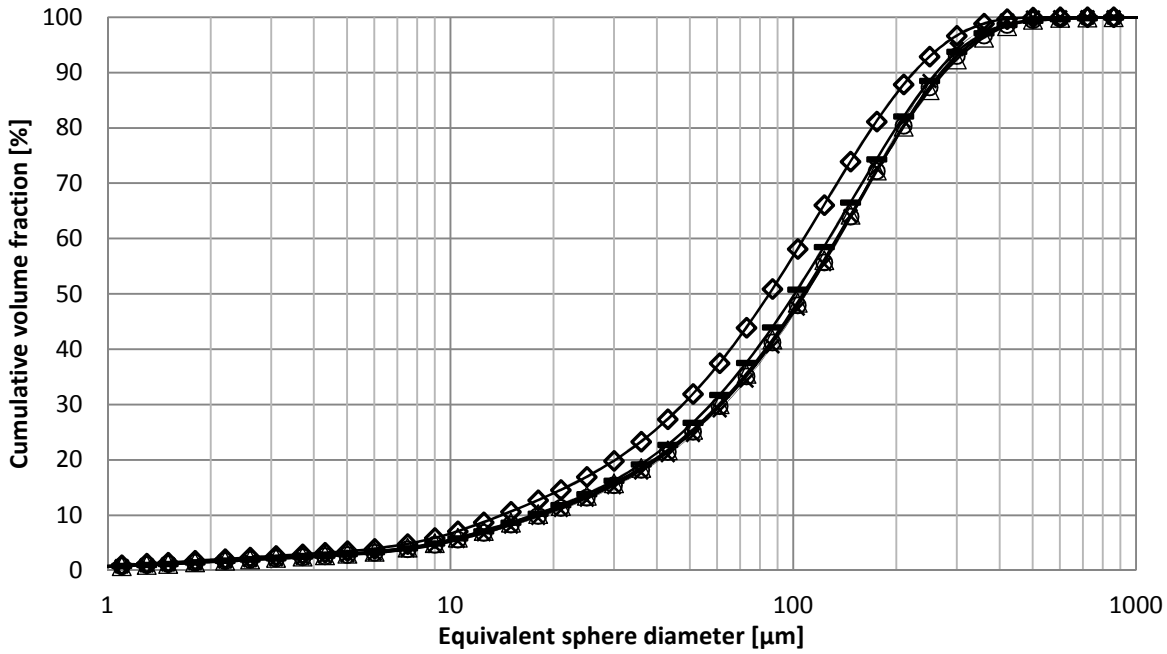

Figure 2: Volume based cumulative distribution of particle size in ERM-BF439a (○), ERM-BF439b (◇), ERM-BF439c (△), ERM-BF439d (-) and ERM-BF439e (x) analysed by laser diffraction (N = 5, n = 2). The total particle volume for each preparation is set as 100 %.

3.3 Total DNA content of the base materials

In order to investigate if both materials used for the production of ERM-BF439 contain the same mass of DNA, a slight modification of the classical fractionation method developed initially by Ogur and Rosen [11] was employed.

A sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds, followed by acidic extraction with 0.84 mol/L perchloric acid (pH 0.3) at 70 °C was performed. The mass of DNA was determined after derivatisation with diphenylamine using a spectrophotometer. Diphenylamine reacts specifically with 2-deoxyribose linked to purine nucleobases to produce a blue-coloured compound that absorbs at 600 nm [11, 12]. The extractable DNA mass fraction of the two materials was calculated as:

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

The ratio of the DNA mass extractable from 100 mg of GM and non-GM maize powder was found to be (1.005 ± 0.008) (N = 9 with an expanded uncertainty, k = 2). A t-test showed, with 95 % confidence, that there was no significant difference between the DNA mass extracted from the GM and non-GM maize powders using the modified Ogur and Rosen method.

The integrity of the DNA was checked by gel electrophoresis. DNA was extracted from 200 mg samples taken from each of the candidate CRM, ERM-BF439a, ERM-BF439b, ERM-BF439c, ERM-BF439d and ERM-BF439e, using a CTAB DNA extraction method (Annex A). None of the samples showed DNA degradation (data not shown).

3.4 Consistency measurements

As a control for the gravimetric preparations, the mass fraction of 4114 maize event in the mixed materials ERM-BF439c, ERM-BF439d and ERM-BF439e was measured using the in-house validated real-time PCR method provided by Pioneer and delivered under confidentiality agreement to IRMM.

At the IRMM, the genomic DNA was extracted by a validated CTAB extraction method (Annex A) using 200 mg powder samples. After the extraction, the DNA 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 maize-specific gene and the transgene. The real-time PCR test was calibrated with genomic DNA extracted from pure 4114 maize powder. For the calibration curve of the maize-specific gene, the DNA was used undiluted (approximately 400 ng DNA per 25 μL reaction) and diluted up to 200-fold. For the calibration curve of the transgene, the DNA was used undiluted (approximately 400 ng DNA per 25 μL reaction) and diluted up to 10000-fold. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of 4114 maize event and from the Cq-values. The LOD of the PCR method was calculated as 3.3-fold *s* of the lowest calibration point at which s_{rel} was below 25 %. The results of the quantification of 4114 maize event are shown in Table 3. The real-time PCR measurements confirmed that the mass fractions of 4114 maize in the mixed materials ERM-BF439c, ERM-BF439d and ERM-BF439e were consistent with the gravimetrical approach used for their preparation. Although no independent calibration was carried out, the data in Table 3 can be used for confirmation of the processing, but do not necessarily represent the true value of the mass fractions.

Table 3: Quantification of the 4114 maize mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure 4114 maize seed powder for calibration

Candidate CRM	4114 maize mass fraction [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF439a	< 0.06 [1) 2)]	-
ERM-BF439b	1063 [1)]	37
ERM-BF439c	0.91 [3)]	0.06
ERM-BF439d	8.6 [4)]	0.4
ERM-BF439e	90.1 [1)]	8.7

1) Mean of 2 samples (extraction replicates) from each of 5 randomly selected bottles (*N* = 5, *n* = 2), with each sample measured in 3 real-time PCR replicates.

2) The value was below the LOD determined during method validation (0.06 g/kg).

3) Mean of 3 samples (extraction replicates) from each of 12 randomly selected bottles (*N* = 12, *n* = 3), with each sample measured in 3 real-time PCR replicates.

4) Mean of 2 samples (extraction replicates) from each of 12 randomly selected bottles (*N* = 12, *n* = 2), with each sample measured in 3 real-time PCR replicates.

4 Homogeneity

A key requirement for any certified reference material aliquotted into units is the equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty associated with the certified value, although it is not necessarily relevant whether the variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1] 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 of the CRMs (Sections 3.4 and 5.1). These data were appropriate for investigating homogeneity since they had been obtained under intermediate precision conditions on bottles taken randomly from the entire batch and analysed in a randomised order. Two extraction replicates per bottle were analysed for ERM-BF439d and ERM-BF439e, compared to three for ERM-BF439c. The number of extraction replicates was chosen based on the intermediate precision of the in-house validated method, such that the standard uncertainty for the within-unit variation would be less than 25 %. Homogeneity of the blank powder was demonstrated in the course of the test for the genetic purity of the raw materials by taking two extraction replicates from 5 randomly selected bottles of ERM-BF439a. The homogeneity of ERM-BF439b is related to the purity study of the seeds. As all 207 tested seeds gave a signal for the 4114 maize event, using the statistical analysis (Poisson distribution for rare events) with 95 % level of confidence the batch was considered to be homogeneous (Section 3.1).

4.1 Between-unit homogeneity

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

For the between-unit homogeneity test, the number of bottles selected corresponds to approximately the cube root of the total number of bottles produced. Therefore, 12 bottles were selected for ERM-BF439c and 14 for ERM-BF439d. To facilitate both the homogeneity studies and the short-term stability study, 15 bottles were selected for ERM-BF439e. For each candidate CRM, a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12, 14 and 15 groups respectively (with a similar number of bottles) and one bottle was randomly selected from each group. For ERM-BF439c, three independent samples (extraction replicates) were taken from each bottle whilst for the candidate CRMs with higher mass fractions, ERM-BF439d and ERM-BF439e, two independent samples (extraction replicates) were taken from each bottle. All samples were 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 trend 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 filling sequence. No trends were observed at a 95 % confidence level.

In addition, regression analyses were performed to evaluate potential trends in the analytical sequence. Some significant trends (95 % confidence level) in the analytical sequence were detected for ERM-BF439d and ERM-BF439e, indicating a trend in the analytical system. The correction for this trend, even if it is statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [13]. Correction of trends is therefore expected to improve the sensitivity of the 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:

$$x_{i\_corr} = x_i - b \cdot i$$ **Equation 1**

b = slope of the linear regression

i = position of the result in the analytical sequence

The data set for ERM-BF439c and the trend-corrected datasets for ERM-BF439d and ERM-BF439e were assessed for consistency using Grubbs outlier tests at a 99 % confidence level on the individual results and on the unit means. No outlying individual results neither unit means were detected using the double Grubbs outlier test.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method intermediate precision, if the individual samples were representative of the whole unit.

Evaluation by ANOVA requires mean values per unit which follow at least a unimodal distribution and results for each unit that have approximately the same standard deviation. Too few data are available for the unit means to make a clear statement about the distribution. Therefore, it was checked visually whether all individual data followed a unimodal distribution using histograms and normal probability plots.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean squares 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 inhomogeneity that could be hidden by method intermediate precision, was calculated as described by Linsinger *et al.* [14]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method intermediate precision ($s_{wb,rel}$), between-unit standard deviation ($s_{bb,rel}$) and maximum hidden inhomogeneity ($u^*_{bb,rel}$) were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}}$$ **Equation 2**

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$ **Equation 3**

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

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

The results of the evaluation of the between-unit variation are summarised in Table 4.

Table 4: Results of the homogeneity study

Candidate CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF439c	18.9	n.c.[1)]	5.9
ERM-BF439d	11.0	n.c.[1)]	4.8
ERM-BF439e	8.2	n.c.[1)]	3.5

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

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation can be used as an estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used for analysis. Using sample sizes equal or above the minimum sample intake guarantees the certified value within its stated uncertainty.

Homogeneity and stability experiments were performed using a 200 mg sample intake. This sample intake gives acceptable intermediate precision, demonstrating that the within-unit inhomogeneity no longer contributes to the analytical variation at this sample intake.

ERM-BF439a and ERM-BF439b are pure non-GM and GM materials, respectively. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. However, based on the real-time PCR measurements carried out on these two powders it was concluded that also for these two pure materials the suitable minimum sample intake for real-time PCR is 200 mg.

5 Stability

Time, temperature and light were regarded as the most relevant influences on the stability of the materials. The influence of light was minimised by storing the materials in containers which minimised light exposure. In addition, materials were stored in the dark and dispatched in boxes, thus removing any possibility of degradation due to light. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for the dispatch of the materials to the customers (short-term stability). During transport, especially in the summer, temperatures of up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The ERM-BF439e material was selected for the short-term stability study because it is the highest concentration mixture of both GM and non-GM base materials, which makes it possible to assess the stability of both base materials. The short-term stability study was carried out using an isochronous design [15]. In this approach, samples of ERM-BF439e were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under intermediate precision conditions.

ERM-BF439 is a dried maize powder, which has been prepared in a similar manner to previous GMO CRM maize powders produced by IRMM and which have similar water content and particle size distribution. Therefore, the data obtained from the stability monitoring of previous maize GMO CRMs were used to assess the long-term stability of ERM-BF439, and to estimate the uncertainty associated with storage for this CRM.

5.1 Short-term stability study

For the short-term stability study, units of ERM-BF439e were stored at 4 °C, 18 °C and 60 °C for each of 1, 2 and 4 weeks, whereupon they were moved to the reference temperature (-70 °C). Units representing the time point of 0 weeks were kept at a reference temperature (-70 °C). Five units per storage time and temperature were selected using a random stratified sampling scheme. From each unit, two extraction replicates were measured by real-time PCR. The measurements were performed under intermediate precision conditions with respect to the PCR plates, and a randomised sequence was used to differentiate any potential analytical trend from a trend over storage time.

The data were evaluated individually for each of the three temperatures tested. The results were screened for outliers using the single and double Grubbs test at a 99 % confidence level. No statistical outliers were detected in any of the studies for any of the temperatures.

Also, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated to test for potential increases/decreases of the 4114 maize mass fraction due to the simulated shipping conditions. The slopes of the regression lines were tested for statistical significance. None of the trends was statistically significant on a 95 % confidence level for any of the temperatures.

The material can thus 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. Previously released maize powder CRMs were analysed for their GM mass fraction with 53 data points over a period of 10 years. On each occasion, measurements were performed simultaneously on one PCR plate, using DNA extracted from units stored at the normal storage temperature (4 °C) and at a reference temperature (-70 °C). Each of these studies can be viewed as a two-point isochronous study. The evaluation was based on the GM mass fraction ratio of results of the samples stored at 4 °C and -70 °C.

To verify that the data obtained from stability monitoring of other maize GMO CRMs produced and stored in the same way as ERM-BF439, can be used to estimate the stability uncertainty contribution for ERM-BF439, the data of the 4 °C short-term stability study (Section 5.1) were compared to the stability monitoring data. The outcome did not contradict the conclusions drawn from the long-term stability study on the uncertainty contribution relating to the storage of the CRM.

The long-term stability data were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No statistical outliers were detected, and the results were retained for the estimation of u_{lts}.

The data were also evaluated against storage time and regression lines were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected at a 95 % confidence level.

The material can, therefore, 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 entirely rule out the degradation of materials, 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 that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation.

The uncertainties of stability during dispatch and storage were estimated, as described in [16]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 5}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 6}$$

s_{rel} relative standard deviation of all results of the stability study

t_i time elapsed at time point *i*

$\bar{t}$ mean of all t_i

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. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for 1 week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the stability monitoring program for maize GMO CRMs. The uncertainty contribution describes the possible degradation during 24 months storage at 4 °C.

The results of these evaluations are summarised in Table 5.

Table 5: Uncertainties of stability during dispatch and storage. $u_{sts,rel}$ was calculated for a temperature of 60 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of 4 °C and 24 months

Candidate CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-BF439	1.5	0.8

After the certification study, the materials will be included in the IRMM's regular stability monitoring programme, to assess their further stability.

6 Characterisation

For the purpose of RM certification, material characterisation is the term used to describe the process of determining the certified value of a reference material.

The five candidate CRMs, under the label ERM-BF439, are maize powder materials processed from non-GM and GM seeds. While ERM-BF439a was prepared from the pure blank material and ERM-BF439b from the pure GM material, the other candidate CRMs of the ERM-BF439 series are gravimetrically diluted mixtures of the pure non-GM and GM seed powders. ERM-BF439 is certified for the mass fraction of 4114 maize event. Gravimetric mixing was chosen as the method of choice based on a primary method of measurement confirmed by PCR analysis.

6.1 Genetic purity of the base materials

The genetic purity with respect to the 4114 maize event of the GM and non-GM batches used for the processing of the candidate CRMs was investigated to calculate the certified value.

No indication was found that the GM maize material contained seeds that were negative for the event 4114 maize (Section 3.1).

The powder used for the production of ERM-BF439a did not contain traces of 4114 maize above the LOD of the real-time PCR method used (Sections 3.1 and 3.4). The certified value for ERM-BF439a is therefore based on the LOD of the real-time PCR method used, as determined during in-house method validation.

The eventual adventitious presence of other GM events in both the GM and non-GM maize powders was verified by using a real-time PCR-based ready-to-use multi-target analytical system for GM detection developed by the JRC Institute for Health and Consumer Protection (IHCP) [17]. This test was performed at the IRMM by using a pre-spotted 96-well plate containing primers and probes for the event-specific simultaneous detection of 44 single-insert GMOs and the primers and probes for the specific detection of the 4 different taxonomic groups:
for maize: HMG, E3272, E98140, BT11, BT176, DAS40278, DAS59122, GA21, MIR162, MIR604, MON810, MON863, MON87460, MON88017, MON89034, NK603, T25, TC1507;
for soya: LEC, A2704, A5547, CV127, DP305423, DP356043, FG72, GTS40-3-2, MON87701, MON89788;
for rapeseed: CruA, GT73, MS1, MS8, RF1, RF2, RF3, T45, Topas19/2;
and for cotton: Sah7, E281, E3006, GHB119, GHB614, LLCotton25, MON1445, MON15985, MON531, MON88913, T304.
Stacked events derived from the single-insert GMOs included in the system would also be detected.
The results indicated that both maize powders used for the production of ERM-BF439 did not contain any of the above tested GM events and were only positive for the taxon-specific detection for maize (HMG).

Since no evidence of contamination was found in both base materials, 100 % genetic purity was used for the calculation of the certified mass fraction of 4114 maize in the powder mixtures. The difference between the statistically established genetic purity of 98.6 % (Section 3.1) and the 100 % genetic purity was taken into account in the uncertainty calculation.

6.2 Mass fractions and their uncertainties

The certified mass fraction values are based on the mass fractions of mixed GM and non-GM powder, corrected for their water mass fractions and taking into account the powder's genetic purity with regards to the 4114 maize event. The values were calculated according to the following equations:

$$\text{GM mass fraction [g/kg]} = \frac{m_{\text{GM, dry}}}{m_{\text{GM, dry}} + m_{\text{nonGM, dry}}} \times 1000 \qquad \textbf{Equation 7}$$

$$m_{\text{GM, dry}} = m_{\text{GM}} \times (1 - \text{WMF}_{\text{GM}}) \qquad \textbf{Equation 8}$$

$$m_{\text{nonGM, dry}} = m_{\text{nonGM}} \times (1 - \text{WMF}_{\text{nonGM}}) \qquad \textbf{Equation 9}$$

$m_{\text{GM,dry}}$ mass [g] of the GM powder corrected for its water mass fraction
$m_{\text{nonGM,dry}}$ mass [g] of the non-GM powder corrected for its water mass fraction
m_{GM} mass [g] of the GM powder used for the dilution
m_{nonGM} mass [g] of the non-GM powder used for the dilution
WMF_{GM} water mass fraction of the GM powder [g/g]
$\text{WMF}_{\text{nonGM}}$ water mass fraction of the non-GM powder [g/g]

The data supporting the calculation of the mass fractions of 4114 maize are summarised in Table 6.

Table 6: Subsequent mixing of GM 4114 maize seed powder with non-GM powder to prepare the ERM-BF439c, d and e materials

Candidate CRM	GM powder [1]			Non-GM powder [1]		Mixtures
	Mass fraction [g/kg]	Water mass fraction ± U (k = 2) [g/kg]	Mass [g]	Water mass fraction ± U (k = 2) [g/kg]	Mass [g]	Calculated GM mass fraction [g/kg]
ERM-BF439e	1000.0 [2]	18.9 ± 2.4	398.60	20.1 ± 2.5	3600.40	100
ERM-BF439d	100.0 [3]	20.9 ± 2.6	400.00	20.1 ± 2.5	3599.80	10.0
ERM-BF439c	10.0 [4]	21.0 ± 2.6	400.00	20.1 ± 2.5	3599.60	1.00

[1] Calculations of the mass fraction of 4114 maize in the powder mixtures are based on a 100 % genetic purity with regard to 4114 maize of the non-GM and GM base materials.

[2] Pure GM powder ERM-BF439b was used for the preparation of ERM-BF439e.

[3] GM powder mixture ERM-BF439e was used for the preparation of ERM-BF439d.

[4] GM powder mixture ERM-BF439d was used for the preparation of ERM-BF439c.

The uncertainties of the certified mass fractions (u_{char}) of the 4114 maize event have several components, i.e. the uncertainty arising from weighing ($u_{\text{char,1}}$), the uncertainty from the determination of the water mass fraction ($u_{\text{char,2}}$), and the uncertainties associated with the determination of the genetic purity with regard to the 4114 maize event of the non-GM and GM base powders ($u_{\text{char,3}}$ and $u_{\text{char,4}}$, respectively). Based on a statistical analysis of the probability distribution of finding a negative seed in the GM base material, it was concluded that the genetic purity of the event 4114 maize event in this CRM, was higher than 98.6 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value (Table 7).

Table 7: Uncertainty budgets for the mass fractions of 4114 maize in ERM-BF439

Candidate CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]				Combined standard uncertainty u_{char} [g/kg]
		$u_{char,1}$ [1)]	$u_{char,2}$ [2)]	$u_{char,3}$ [3)]	$u_{char,4}$ [4)]	
ERM-BF439a	0	n.a.[5)]	n.a.[5)]	0.0173	n.a.[5)]	0.0173
ERM-BF439b	1000	n.a.[5)]	n.a.[5)]	n.a.[5)]	4.1767	4.1767
ERM-BF439c	1	0.0044	0.0026	0.0173	0.0042	0.0185
ERM-BF439d	10	0.0360	0.0227	0.0173	0.0416	0.0620
ERM-BF439e	100	0.2549	0.1852	0.0173	0.4168	0.5228

[1)] Standard uncertainty of the mass determination, based primarily 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 genetic purity estimation of the non-GM base material (LOD = 0.06 g/kg), based on the half-width of the interval between 0 and 0.06 g/kg, divided by the square root of 3 (rectangular distribution).

[4)] Standard uncertainty of the genetic purity estimation of the GM base material (> 98.6 %), based on the interval between 98.6 % and 100 % divided by the square root of 3 (rectangular distribution).

[5)] n.a. - not applicable

6.3 Verification measurements

Real-time PCR measurements demonstrated that no mixing errors were made (Section 3.4). Gel electrophoresis proved that the DNA was not degraded during processing of the candidate CRMs (Section 3.3).

7 Value Assignment

Certified values are values that fulfil the highest standards of accuracy assessment. Therefore full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established.

The assigned certified values are based on the masses of dried powder of GM seeds and non-GM 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 6).

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} (Section 6.2), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5.3). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,rel}$) with a coverage factor k given as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{char,rel}^2 + u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2} \qquad \textbf{Equation 10}$$

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

For the blank material, the LOD of the method was used to describe the 95 % confidence interval of the certified mass fraction of the event (< 0.06 g/kg). This was supported by the high genetic purity with regards to the 4114 maize event of the non-GM material and the absence of a mixing step; calculating the U_{CRM} for the blank material on the basis of the only quantifiable standard uncertainty ($u_{char,3}$) gives a value of U = 0.04 g/kg (assuming k = 2), which is below the certified < 0.06 g/kg value. The LOD is, therefore, a conservative estimate of the certified value and its uncertainty.

For the pure GM material, the statistically calculated genetic purity of the GM seed batch (Section 3.1) was used to describe the 95 % confidence interval of the certified mass fraction of the event (> 986 g/kg). Calculating U_{CRM} for the pure GM material on the basis of the only quantifiable standard uncertainty ($u_{char,4}$) gives a value of U = 8 g/kg (assuming k = 2), which is less than the difference between the nominal value (1000 g/kg) and the certified value (> 986 g/kg). The statistically calculated genetic purity is, therefore, a conservative estimate of the certified value and its uncertainty.

For the three mixtures, the certified values were established by gravimetry, and the measured mass fraction values had an expanded uncertainty with a coverage factor of 2, established during calibration of the balance. Therefore, the same coverage factor (k = 2) was used to obtain the expanded uncertainties for ERM-BF439c, d and e.

The certified values and their uncertainties are summarised in Table 8.

Table 8: Certified values and their uncertainties for ERM-BF439

CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{lts} [g/kg]	U_{CRM} [3)] [g/kg]
ERM-BF439a	< 0.06 [1)]	0.0173	n.a.[4)]	n.a.[4)]	n.a.[4)]	-
ERM-BF439b	> 986 [2)]	4.1767	n.a.[4)]	n.a.[4)]	n.a.[4)]	-
ERM-BF439c	1.00	0.0185	0.0588	0.0149	0.0080	0.13
ERM-BF439d	10.0	0.0620	0.4786	0.1496	0.0798	1.1
ERM-BF439e	100	0.5228	3.4925	1.4968	0.7983	8

[1)] With 95 % confidence, the certified value is below this level.

[2)] With 95 % confidence, the certified value is above this level.

[3)] Expanded (k = 2) and rounded uncertainty

[4)] n.a.- not applicable

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The certified identity is based on the documentary traceability to the 4114 maize event application, given in the patent (publication number US20110154523 A1) [8].

Quantity value

The traceability chain for ERM-BF439c, d and e is based on the use of calibrated balances and a thorough control of the weighing procedure.

The certified values for the pure non-GM and GM CRMs, ERM-BF439a and ERM-BF439b, respectively, are based on the genetic purity assessment using event-specific 4114 maize real-time PCR.

The certified values are therefore traceable to the International System of units (SI).

8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent whole measurement process. Often the complete identity of these 'intermediate analytes' is not entirely known or taken into account. Therefore, it is difficult to mimic all 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 summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the CLSI Guideline C53-A [18] 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 is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is established, the results from routinely used methods can be legitimately compared with the certified value to determine whether a bias does exist in calibration, and the CRM can be used as a calibrant.

The CRM ERM-BF439 was prepared from non-GM and GM maize seed powder and therefore the analytical behaviour will be the same as for a routine sample of milled maize seeds/grains. For other types of samples the commutability has to be assessed.

9 Instructions for use

9.1 Safety and protection of the environment

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

9.2 Storage conditions

The materials should be stored at (4 ± 3) °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material is hygroscopic. The user should close CRM 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 for opened bottles.

9.3 Minimum sample intake

The minimum sample intake for a DNA extraction is 200 mg maize powder.

ERM-BF439a and ERM-BF439b are pure non-GM and GM materials. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. Nevertheless it is recommended that the same sample intake is used as for the mixed materials to obtain a significant amount of DNA.

9.4 Use of the certified value

The main purpose of these materials is for calibration or quality control of 4114 maize event identification and quantification methods. As with any reference material, they can be used for establishing control charts and validation studies.

The user is reminded that this reference material is certified for its 4114 maize mass fraction and should be used for measurements expressed in mass fractions. The exact relationship between the certified GM powder mass fractions and the corresponding DNA copy number ratio is not known. Changing the measurement unit from mass fraction to copy number per haploid genome equivalent, for instance, requires the use of a conversion factor that is only an approximate value, thereby adding additional uncertainty to the measurement result.

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 effectiveness of the control, as calibrant and quality control material are based on the same raw materials.

If this is unavoidable, it is recommended that different mass fraction levels of ERM-BF439 are used 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 [19]).

When assessing the method performance, the measured values of the CRMs are compared to the certified values. The procedure is summarised here:

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

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

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Annexes

Annex A: CTAB DNA extraction method (as modified in-house)

Solutions and reagents

1) CTAB buffer A
   - 2 % (w/v) CTAB
   - 1.4 M NaCl
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA pH 8.0
2) CTAB buffer B
   - 1 % (w/v) CTAB
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA, pH 8.0
3) Chloroform:1-Octanol (24:1)
4) 1.2 M NaCl
5) Ethanol
6) 70 % (v/v) Ethanol
7) TE low buffer
   - 1 mM Tris, pH 8.0
   - 0.01 mM Na_2EDTA, pH 8.0
8) Proteinase K, 20 mg/mL
9) RNase A, 100 mg/mL

Protocol

a) Weigh 200 mg powder into a 2 mL microcentrifuge tube.
b) Add 1 mL of CTAB buffer A and mix thoroughly by vortexing.
c) Add 10 µL RNase A and mix by briefly vortexing.
d) Incubate 15 min at 65 °C, mix a few times by shaking
e) Add 20 µL Proteinase K and mix by briefly vortexing
f) Incubate 15 min at 65 °C, mix a few times by shaking
g) Centrifuge 10 min at 13000 x *g* at RT.
h) Transfer the supernatant to a 2 mL microcentrifuge tube containing 500 µL of chloroform:1-octanol (24:1).
i) Vortex 10 sec and centrifuge 10 min at 13000 x *g* at RT.
j) Transfer the upper phase to a new 2 mL microcentrifuge tube containing 700 µL of chloroform:1-octanol (24:1).
k) Vortex 10 sec and centrifuge 5 min at 13000 x *g* at RT.

l) Transfer upper phase to a new 2 mL microcentrifuge tube, carefully determining the volume transferred.

m) Add 2 volumes of buffer B and mix by pipetting up and down.

n) Incubate for 60 min at RT.

o) Centrifuge for 10 min at 20000 x *g* at RT, discard the supernatant by pipetting and conserve the pellet.

p) Add 400 µL of 1.2 M NaCl and vortex gently.

q) Add 400 µL of chloroform:1-octanol (24:1), vortex 10 sec and centrifuge 5 min at 13000 x *g* at RT.

r) Transfer upper phase to a new 1.5 mL microcentrifuge tube, carefully determining the volume transferred.

s) Add 2 volumes of cold (-20°C) ethanol to each tube and mix by inverting 10 times.

t) Centrifuge for 10 min at 20000 x *g* at 4 °C.

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

v) Vortex briefly and centrifuge for 5 min at 13000 x *g* at 4 °C.

w) Discard the supernatant by pipetting and air-dry the pellet for about 5 min.

x) Dissolve the DNA pellet in 100 µL of TE low buffer preheated to 50 °C.

y) Incubate for 10 min at 50 °C, shaking at 350 rpm.

z) Let the pellet dissolve completely overnight at 4 °C. Store at +4 °C (short term) or -20 °C (long term).

Annex B: Results of the homogeneity measurements

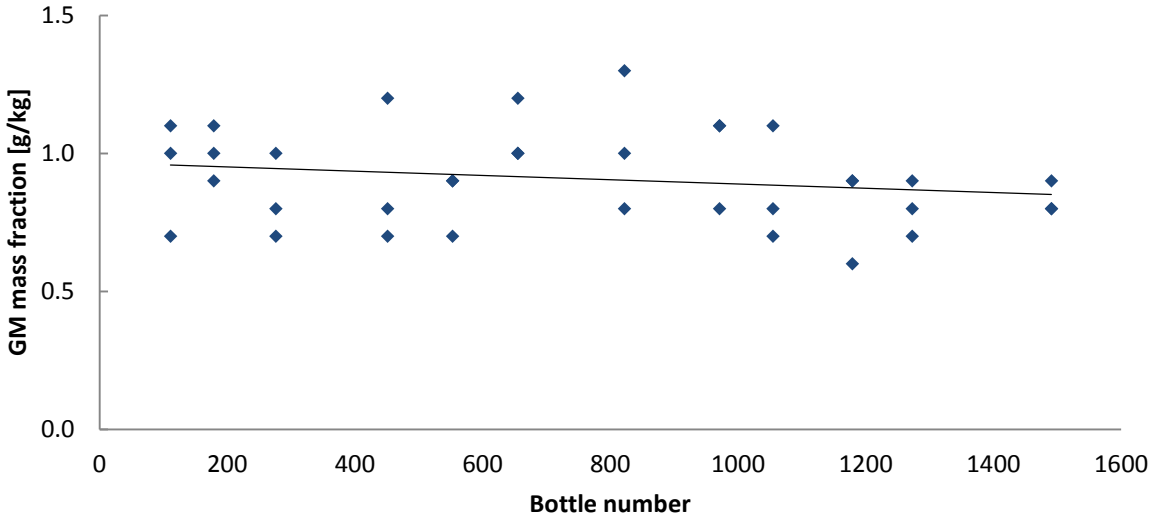

Figure B1: Real-time PCR measurement results for ERM-BF439c. Three samples (extraction replicates) were measured from each of 12 randomly selected bottles (*N* = 12, *n* = 3), with each sample measured in 3 real-time PCR replicates. The straight line is a least-squares linear regression through all data points.

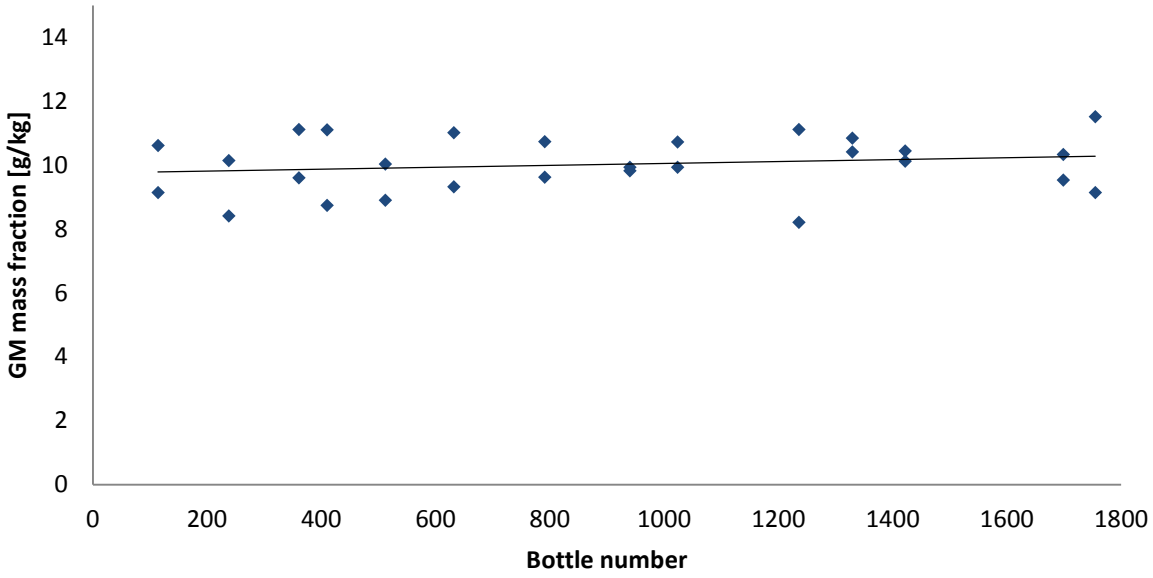

Figure B2: Real-time PCR measurement results for ERM-BF439d. Two samples (extraction replicates) were measured from each of 14 randomly selected bottles (*N* = 14, *n* = 2), with each sample measured in 3 real-time PCR replicates. The straight line is a least-squares linear regression through all data points.

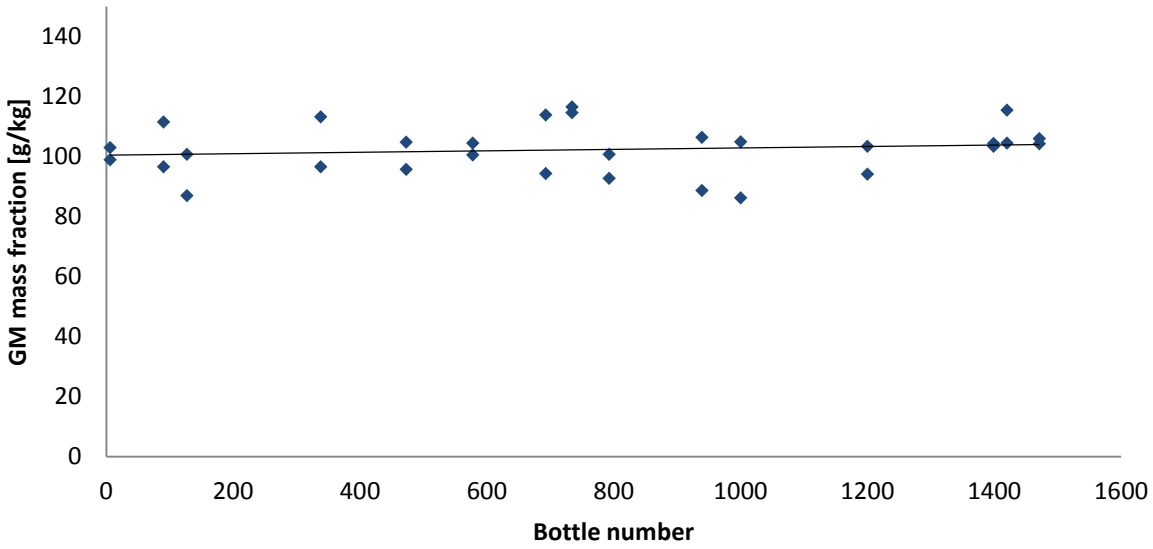

Figure B3: Real-time PCR measurement results for ERM-BF439e. Two samples (extraction replicates) were measured from each of 15 randomly selected bottles (N = 15, n = 2), with each sample measured in 3 real-time PCR replicates. The straight line is a least-squares linear regression through all data points.

Annex C: Results of the short-term stability measurements

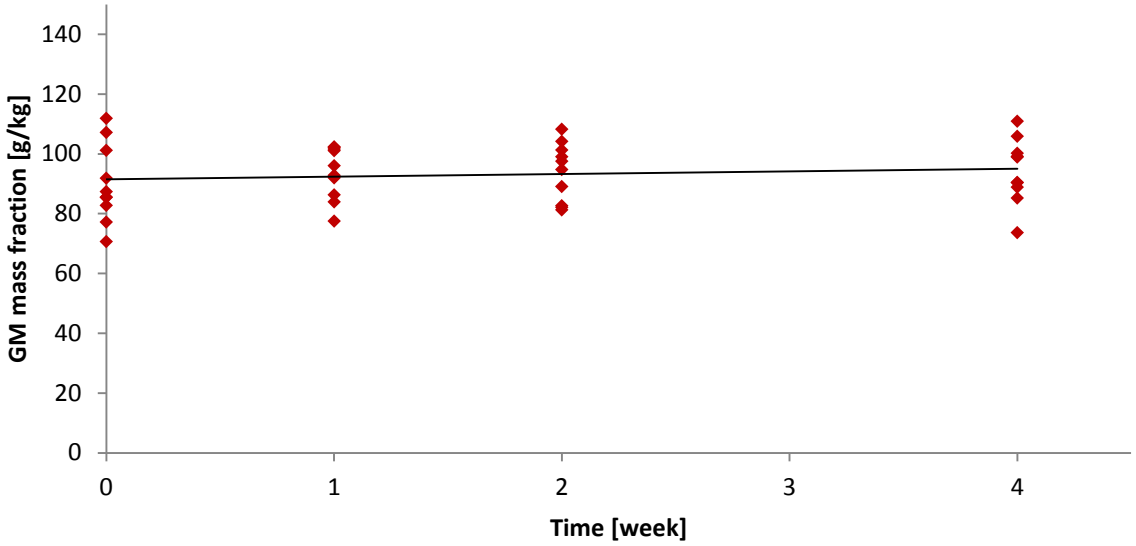

Figure C1: Real-time PCR measurement results for ERM-BF439e during short-term stability testing at 4 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates. The straight line is a least-squares linear regression through all data points.

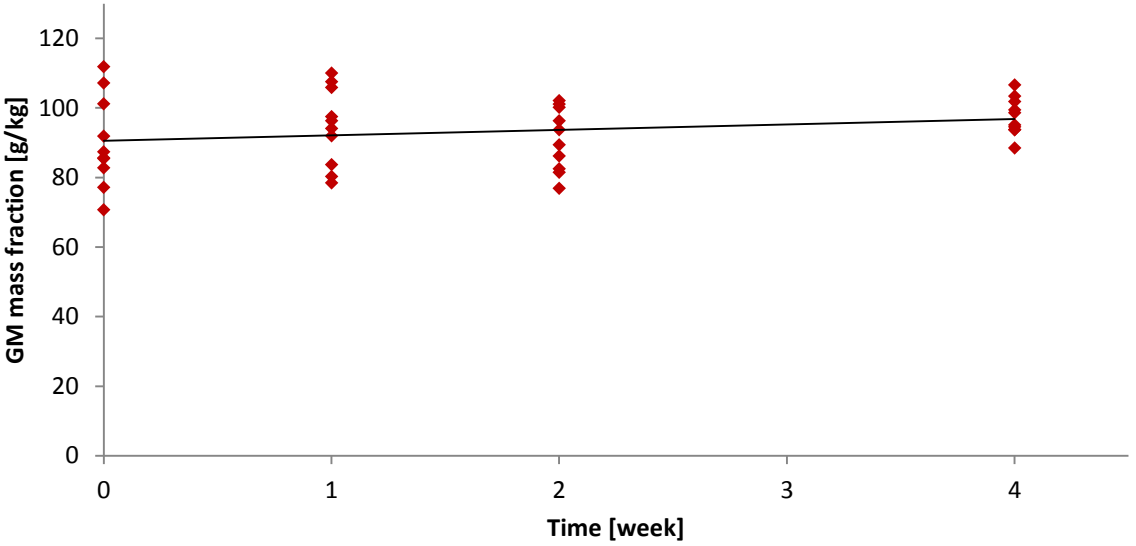

Figure C2: Real-time PCR measurement results for ERM-BF439e during short-term stability testing at 18 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates. The straight line is a least-squares linear regression through all data points.

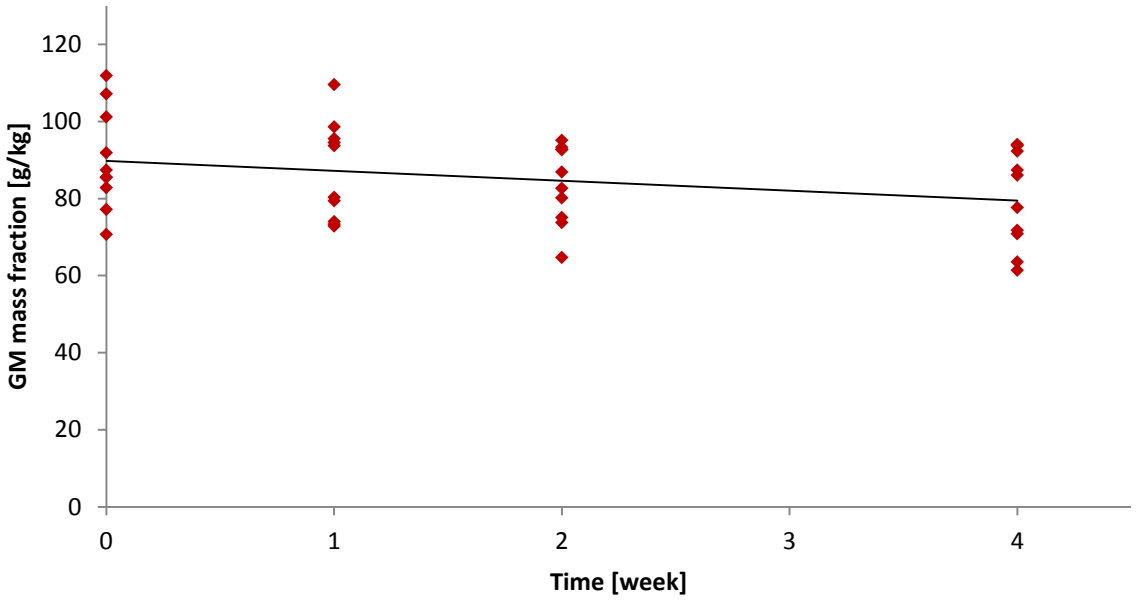

Figure C3: Real-time PCR measurement results for ERM-BF439e during short-term stability testing at 60 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates. The straight line is a least-squares linear regression through all data points.

Annex D: Results of the long-term stability measurements

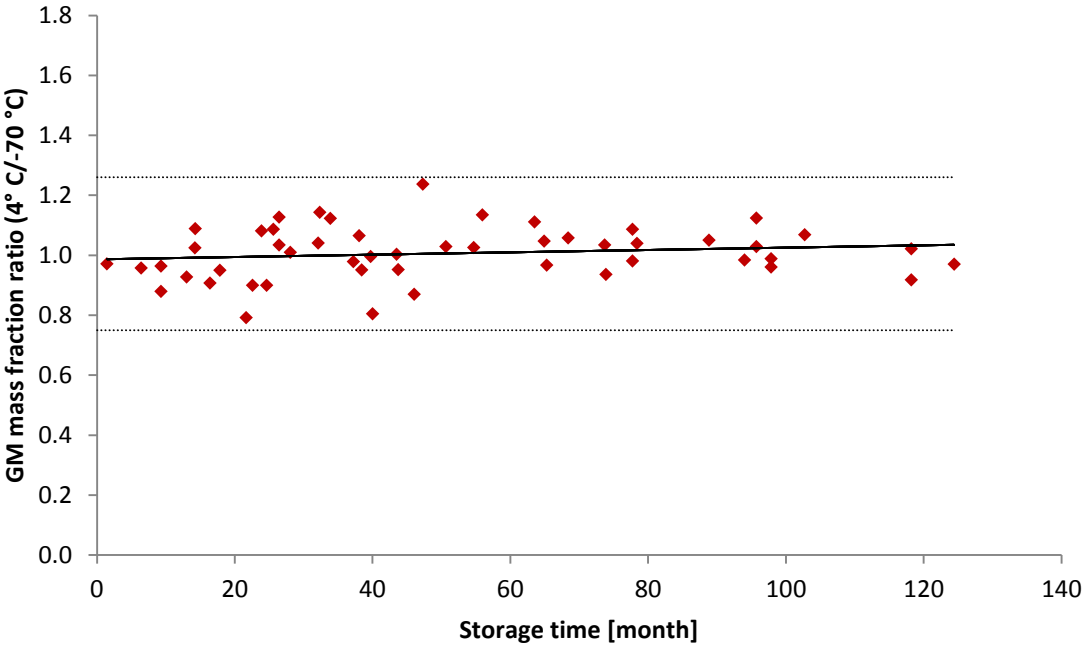

Figure D1: Real-time PCR measurement results for ERM-BF411, ERM-BF412, ERM-BF413, ERM-BF413k, ERM-BF414, ERM-BF415, ERM-BF416, ERM-BF417, ERM-BF418, ERM-BF420, ERM-BF424, ERM-BF427 and ERM-BF433 during post-certification monitoring. The dashed lines give the limits of 3 *s* obtained for the measurement results. The straight line is a least-squares linear regression through all data points.

European Commission

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Certified Reference Materials ERM®-BF439a, ERM®-BF439b, ERM®-BF439c, ERM®-BF439d and ERM®-BF439e

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