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European Reference Materials

CERTIFICATION REPORT

The certification of different mass fractions of MON-Ø4Ø32-6 in soya bean powder

Certified Reference Materials ERM®-BF410dn and ERM®-BF410gn

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Abstract
This report describes the production of two Certified Reference Materials (CRMs), ERM BF410dn and ERM-BF410gn, which are certified for their GTS 40-3-2 soya bean (unique identifier MON-Ø4Ø32-6) mass fractions. These materials were produced following ISO Guide 34:2009 and are certified in accordance with ISO Guide 35:2006.
During the production of ERM-BF410k (released in 2008), the genetically modified (GM) soya bean seeds (GM event GTS 40-3-2) and seeds from a non-GM soya bean were milled to obtain GM and non-GM seed powders with a similar particle size distribution. The same powder materials were used for the production of ERM-BF410n.
The certified values were obtained from the gravimetric preparations, taking into account the water mass fractions of the two powder materials and the genetic purity with respect to the GTS 40-3-2 soya bean (genetic purity data were taken from the previous production). 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 qPCR measurements to identify GTS 40-3-2 soya bean and 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 soya bean 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 panel.

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Certified Reference Materials ERM®-BF410dn and ERM®-BF410gn

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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 two Certified Reference Materials (CRMs), ERM-BF410dn and ERM-BF410gn, which are certified for their GTS 40-3-2 soya bean (unique identifier MON-Ø4Ø32-6) mass fractions. These materials were produced following ISO Guide 34:2009 [1] and are certified in accordance with ISO Guide 35:2006 [2].

During the production of ERM-BF410k (released in 2008), the genetically modified (GM) soya bean seeds (GM event GTS 40-3-2) and seeds from a non-GM soya bean were milled to obtain GM and non-GM seed powders with a similar particle size distribution. The same powder materials were used for the production of ERM-BF410n.

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

The following values were assigned:

	GTS 40-3-2 soya bean mass fraction [1)]	
	Certified value [g/kg]	Uncertainty [g/kg] [3)]
ERM-BF410dn	10.0 [2)]	1.0
ERM-BF410gn	100 [2)]	7

[1)] Genetically modified soya bean with the unique identifier MON-Ø4Ø32-6.

[2)] This certified value is based on the masses of dried genetically modified GTS 40-3-2 soya bean powder and dried non-modified soya bean powder that were mixed, taking into account their respective genetic purity with regard to GTS 40-3-2 soya bean and their respective water content. The certified value is traceable to the International System of Units (SI).

[3)] 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
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EC	European Commission
ERM®	Trademark of European Reference Materials
EU	European Union
EU-RL 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
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
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 the placing on the market of 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 are also required 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.

Monsanto (St. Louis, Missouri, US) developed the genetically modified (GM) GTS 40-3-2 soya bean event (unique identifier code MON-Ø4Ø32-6, following Commission Regulation (EC) No 65/2004 [7]) as a transgenic herbicide tolerant crop. The GTS 40-3-2 soya bean event was developed by *Agrobacterium tumefaciens* mediated transformation. The event contains the gene coding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that gives the plant resistance to glyphosate, the active ingredient in the herbicide Roundup® [8]. As some of the mass fractions of the previously certified reference material (CRM) ERM®-BF410k (first replacement batch) were almost sold out in 2015, it was decided that a new series of GTS 40-3-2 (Roundup Ready soya bean) reference material was to be produced. This new series received the code ERM-BF410n with 'n' indicating the second replacement batch. It is composed of two CRMs containing different mass fractions of GTS 40-3-2 soya bean (Table 1).

Table 1: Codes indicating the CRMs from the three different productions of ERM-BF410

Nominal conc. (%)	ERM-BF410 (2002)	ERM-BF410k (2008)	ERM-BF410n (2016)
0	ERM-BF410a	ERM-BF410ak	-
0.1	ERM-BF410b	ERM-BF410bk	-
0.5	ERM-BF410c	-	-
1	ERM-BF410d	ERM-BF410dk	ERM-BF410dn
2	ERM-BF410e	-	-
5	ERM-BF410f	-	-
10	-	ERM-BF410gk	ERM-BF410gn
100	-	-	-

1.2 Choice of the material

Seeds were selected as the source of raw material because of their high degree of purity. The two CRMs, ERM-BF410dn and ERM-BF410gn, were produced from powders of GM and non-GM seeds, prepared during the production of the ERM-BF410k series.

1.3 Design of the CRM project

The reported purity and genetic composition of both soya bean seed batches were verified at IRMM in the frame of the previous certification of ERM-BF410 and were reported in ERM-BF410k certification report [9].

Mixtures of non-GM and GM soya bean powder were gravimetrically prepared. The first mixed material ERM-BF410gn was prepared by mixing pure GM with non-GM soya bean powder. ERM-BF410dn was then prepared by further dilution of ERM-BF410gn with non-GM soya bean powder.

The different mass fractions of ERM-BF410dn and ERM-BF410gn 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

Monsanto (St. Louis, Missouri, US) provided the raw materials.

Monsanto initially provided the event-specific qPCR method under a confidential agreement with JRC. Since 2007, it is validated and published by the EU-RL GMFF [10].

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

As described in the ERM-BF410k certification report, Monsanto supplied IRMM in 2001 with non-GM and GM soya bean seeds. According to the information provided by Monsanto, the GTS 40-3-2 soya bean seeds (trade name - Roundup Ready soya bean) are homozygous GM seeds of the variety, AG5602. The non-GM comparator is A1900. In 2007, the seeds were processed into fine powders and a portion of each was used as starting materials for the production of ERM-BF410k. The remainder was stored at -20 °C. In 2015 the CRMs from ERM-BF410k were selling out, and therefore the powder materials (stored at -20 °C) were used as starting materials to produce this ERM-BF410n series. Therefore, the genetic purities with respect to the GTS 40-3-2 event of the delivered GM and non-GM soya bean seeds were taken from ERM-BF410k. The same data are reported in the certification report for the ERM-BF410k [9].

The genetic purity of the GM soya bean seeds was assessed at IRMM by analysing 50 randomly selected seeds for the presence of the GM event GTS 40-3-2. The GM soya bean seeds were germinated on moistened paper in a growing chamber for 15 days. 50 mg pieces from young leaves were sampled from 46 plants. The plant tissues were disrupted in a lysis buffer using the Mixer Mill MM 300 (Retsch, Haan, DE) and 3-mm tungsten carbide beads (Qiagen, Hilden, DE). Genomic DNA was extracted using the DNeasy® 96 Plant kit (Qiagen,

Hilden, DE). The quality of the extracted DNA was analysed on an agarose gel and quantified using the PicoGreen® dsDNA quantitation kit (Molecular Probes Europe, Leiden, NL). The detection of GTS 40-3-2 soya bean event was performed following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [11]. The results showed that all the GM seeds tested gave a signal for the presence of the GTS 40-3-2 soya bean event. Statistical analysis of the 46 measurements (Poisson distribution for rare events) revealed that the GM soya bean seed batch had a genetic purity of > 93.5 % (95 % level of confidence). The calculated genetic purity with respect to the GTS 40-3-2 event of > 93.5 % was taken into account for the estimation of the uncertainties associated to the certified values of the CRMs from this ERM-BF410n production (Section 6.1).

The non-GM seed batches from Monsanto, were also tested at Monsanto for their purity using an enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) for further confirmation of the genetic identity. The company delivered the following information:

- Sixty pools each of 50 seeds of the conventional non-GM soya bean variety A1900 were qualitatively tested by immunoassay for the CP4 EPSPS protein and all were found to be negative.

- Additionally, a GTS 40-3-2 event-specific PCR was performed on the non-GM status of soya bean variety A1900. For the PCR methods aspartate aminotransferase gene (*aat*) was used as a control. For the A1900 seeds, three pools of 50 seeds tested negative by PCR for GTS 40-3-2 and positive for the *aat* gene. These PCR results confirmed the immunoassay results on a genetic level [9].

For the estimation of the uncertainties associated with the certified values of the CRMs from ERM-BF410n production, the genetic purities of the GM batch and the non-GM batch were taken from the ERM-BF410k. For the purity of the non-GM, the qPCR measurement results on DNA samples extracted from the fine non-GM soya bean seed powder with a limit of detection (LOD) of 0.07 g/kg were used. The method did not detect the event GTS 40-3-2 (Section 3.4) which was in agreement with the 99.9 % genetic purity (95 % level of confidence) reported by Monsanto for the delivered non-GM seed material.

3.2 Processing and process control

In 2007, the non-GM and GM seeds were processed separately into powders. Cross-contamination between them and contamination with foreign DNA were avoided by treating all the contact surfaces with DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) before exposure to the materials and using clean laboratory clothing. An in-house validation study had previously shown that the solution degraded DNA effectively under the given conditions.

In the frame of this certification project the water mass fractions of the non-GM powder and the GM powder were measured as (11.5 ± 0.7) g/kg (*U*, *k* = 2) and (6.8 ± 0.03) g/kg (*U*, *k* = 2), respectively (*N* = 1, *n* = 3) (Table 2).

Also, the particle size distribution for both powders was measured based on their deconvoluted laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and were then 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 powder materials was 118.8 µm ± 13.6 µm (*s*) and 115.8 µm ± 11.8 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 2). These size classes were denoted as 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 soya bean powders. It was concluded that the non-GM and GM powder materials were sufficiently similar with respect to their particle size distribution and they could be processed further without introducing a bias which could subsequently affect the extractability of the DNA.

Table 2: The water mass fraction determined by V-KFT and additionally the particle diameter and particle size distribution based on the deconvoluted laser diffraction patterns of the powder materials

Powder material	Water mass fraction [g/kg]		Mean particle diameter [µm]		Particle size distribution X10 [µm]		Particle size distribution X50 [µm]		Particle size distribution X90 [µm]	
	$\bar{x}$	U	$\bar{x}$	s	$\bar{x}$	U	$\bar{x}$	U	$\bar{x}$	U
Non-GM powder	11.5 [1)]	0.7	118.8 [2)]	13.6	19.0 [3)]	2.9	80.6 [3)]	10.3	276.6 [3)]	41.0
GM powder	6.8 [1)]	0.03	115.8 [2)]	11.8	15.2 [3)]	2.3	80.8 [3)]	10.3	271.4 [3)]	40.2

[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 on soya bean powder.

[2)] Mean of one sample (N = 1, n = 5) with the sample standard deviation

[3)] Mean of one sample (N = 1, n = 5). Given are the equivalent sphere diameters for which 10 %, 50 % or 90 % of the particles in 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 measurement.

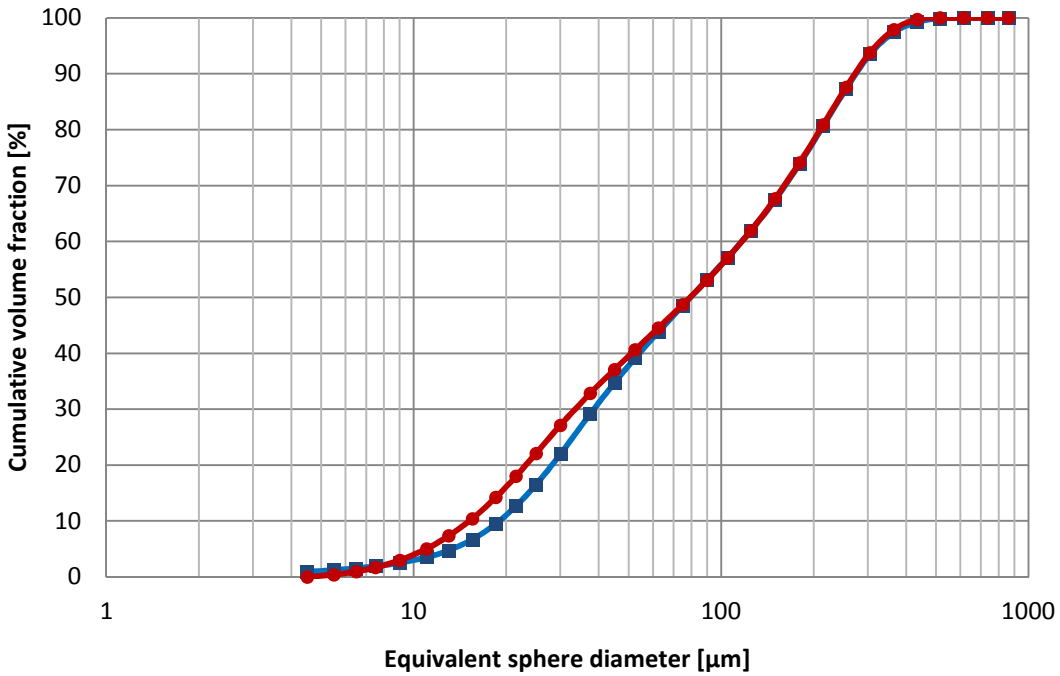

Figure 1: Volume-based cumulative distribution of equivalent sphere diameters in the GM powder (●) and non-GM powder (■) analysed by laser diffraction (N = 1, n = 5). The total particle volume for each material is set as 100 %.

The two mixtures at nominal mass fraction levels of 10 and 100 g/kg of the GTS 40-3-2 soya bean event were prepared using the non-GM and the pure GM GTS 40-3-2 soya bean seed powder materials. 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.

Both materials 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 (MSU-8202-S, Sartorius) with an intermediate precision, determined during calibration and expressed as standard uncertainty (u), of 0.01 g. Calibration of the balance is performed 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 corrected for their respective water content. Portions of the powder materials were weighed into a container and mixed for 1 h by using a Dyna-MIX CM 200 (WAB, Basel, CH). The material with a nominal GTS 40-3-2 soya bean mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM powder materials. Similarly, the material with a nominal GTS 40-3-2 soya bean mass fraction of 10 g/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder. At each mixing step, the water mass fraction of the materials was taken into account. During the certification process, the gravimetric preparation was the basis for the calculation of the certified GTS 40-3-2 soya bean mass fraction for the two powder mixtures (Section 6).

An automatic filling device (All-Fill Sandy, UK) was used to fill the powders into 10 mL brown glass bottles. To avoid cross contamination the equipment was cleaned between the two mass fraction levels and the first 30 bottles of each batch were discarded as an additional precaution. The ERM-BF410dn (nominal 10 g/kg) material was filled first, followed by the ERM-BF410gn (nominal 100 g/kg). Lyophilisation inserts were automatically placed in the bottle necks. The bottles were then placed in a freeze-dryer (Epsilon 2-100D of Martin Christ, Osterode, DE) 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 two mass fraction levels of GTS 40-3-2 soya bean event: nominal 10 g/kg = red (BF410dn) and nominal 100 g/kg = brown (BF410gn), 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 ± 3 °C.

Ten randomly selected bottles from both 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 3.

Table 3: Water mass fraction of candidate ERM-BF410n CRMs determined by V-KFT (N = 10, n = 2). The associated expanded uncertainty (U) has been estimated during validation of the V-KFT method on soya bean powder

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	U (k = 2)
ERM-BF410dn	11.3	1.5
ERM-BF410gn	10.9	1.5

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 both of the candidate CRMs were analysed twice (N = 5, n = 2) and 99.86 % of the particles had a size below 1220 µm (Figure 2). The mean particle diameters and standard deviations of the mean, measured by laser diffraction, were 147 µm ($s_{\bar{x}}$ = 40 µm) and 150 µm ($s_{\bar{x}}$ = 24 µm) for ERM-BF410dn and ERM-BF410gn, respectively.

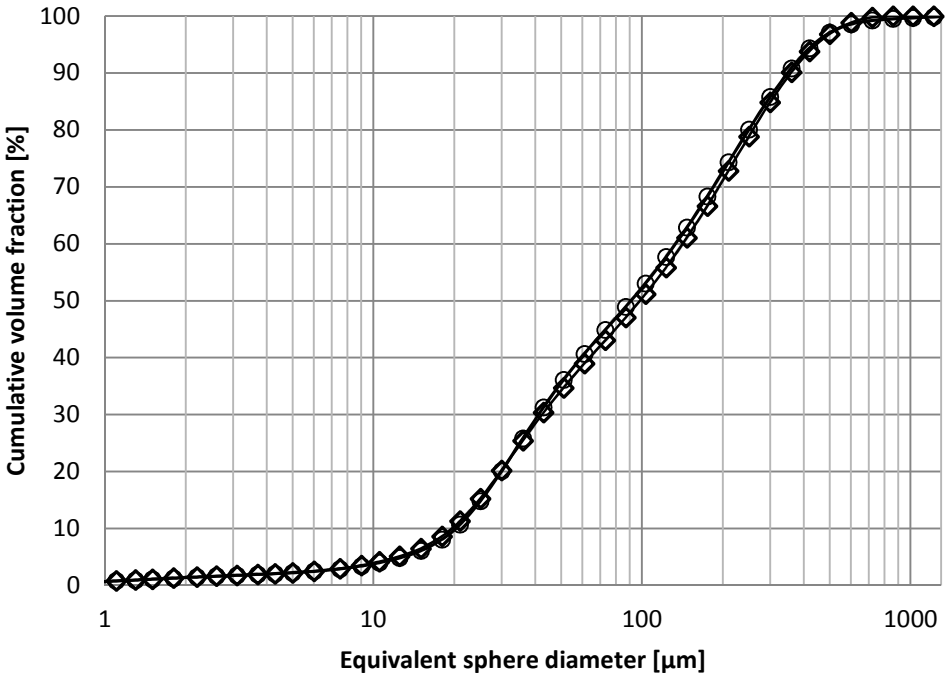

Figure 2: Volume based cumulative distribution of particle size in ERM-BF410dn (○) and ERM-BF410gn (◇) analysed by laser diffraction (N = 5, n = 2). The total particle volume for each preparation is set as 100 %.

3.3 Total DNA content of the powder materials

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 [12] 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 [12, 13]. The extractable DNA mass fraction of the two materials was calculated as:

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

The ratio of the DNA mass extractable from 100 mg of GM and non-GM soya bean powder was found to be (1.10 ± 0.02) (N = 15 with an expanded uncertainty, k = 2). A t-test confirmed a significant difference between the DNA mass extracted from the GM and non-GM powder by the modified Ogur and Rosen [12] method (95 % confidence level).

If the different DNA extractability of GM and non-GM soya bean powders is taken into account, the assigned certified GM mass values of the prepared mixtures ERM-BF410dn and ERM-BF410gn can only be reproduced by real-time PCR. The difference in the extractability

can be attributed to the small difference in the size of the non-GM and GM seeds as observed during their visual inspection. In this context it has to be understood that the ERM-BF410n has been developed to set a common reference point for the implementation of EU legislation on GMO thresholds and labelling.

Gel electrophoresis was used to check the integrity of the DNA. DNA was extracted from 200 mg samples taken from each of the candidate CRM, ERM-BF410dn and ERM-BF410gn, using a CTAB-tip 20 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 GTS 40-3-2 soya bean event in the mixed materials ERM-BF410dn and ERM-BF410gn was measured using the in-house validated real-time PCR method provided by Monsanto. In 2007, this method was validated and published by EU-RL GMFF [10].

At the IRMM, 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 soya bean-specific gene and the transgene. The real-time PCR test was calibrated with genomic DNA extracted from pure GTS 40-3-2 soya bean powder. For the calibration curve of the soya bean-specific gene, the DNA was used undiluted (approximately 200 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 50 ng DNA per 25 µL reaction) and diluted up to 2000-fold. The efficiency of the amplification was assessed from the slope of the regression line between the calibrants' mass fractions of GTS 40-3-2 soya bean 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 GTS 40-3-2 soya bean event are shown in Table 4. The real-time PCR measurements confirmed that the mass fractions of GTS 40-3-2 soya bean in the mixed materials ERM-BF410dn and ERM-BF410gn were consistent with the gravimetrical approach used for their preparation. Although no independent calibration was carried out, the data in Table 4 can be used for confirmation of the processing, but do not necessarily represent the true value of the mass fractions. The event-specific method was used to allow comparison of the results obtained from the new batch ERM-BF410n, and the previous one ERM-BF410k (Annex B). A *t*-test showed that both batches (ERM-BF410k and ERM-BF410n) are sufficiently similar as no significant difference between the measured GM mass fraction values was shown.

Table 4: Quantification of the GTS 40-3-2 soya bean mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure GTS 40-3-2 soya bean seed powder for calibration

Candidate CRM	GTS 40-3-2 soya bean mass fraction [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF410dn	9.9 [1)]	0.3
ERM-BF410gn	95 [2)]	4

[1)] Mean of 2 samples (extraction replicates) from each of 13 randomly selected bottles (N = 13, n = 2), with each sample measured in 3 real-time PCR replicates.

[2)] 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.

4 Homogeneity

A key requirement for any CRM 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-BF410dn and ERM-BF410gn. 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 %. Here we only report on the results of a homogeneity study performed on both mixtures produced within this project, ERM-BF410dn and ERM-BF410gn. Homogeneity of the non-GM and the pure GM GTS 40-3-2 soya bean seed powders used for the production of ERM-BF410n was analysed in the frame of the previous ERM-BF410k production. As all tested seeds gave a signal for the GTS 40-3-2 soya bean 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 CRMs are 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, 13 bottles were selected for ERM-BF410dn. To facilitate both the homogeneity studies and the short-term stability study, 15 bottles were selected for ERM-BF410gn. For both candidate CRMs, a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 13 and 15 groups respectively (with a similar number of bottles) and one bottle was randomly selected from each group. For both candidate CRMs, 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 C.

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. No statistically significant trend was detected, to a 95% confidence level.

The data sets for ERM-BF410dn and ERM-BF410gn 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, nor 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 visually checked 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:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 1}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{\nu_{MSwithin}}}}{\bar{y}} \qquad \textbf{Equation 3}$$

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 5.

Table 5: Results of the homogeneity study

Candidate CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF410dn	7.9	n.c.[1)]	n.c.[1)]	3.5
ERM-BF410gn	4.7	1.0	1.0	2.0

[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.

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 reduce 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-BF410gn material was selected for the short-term stability study because it is the highest concentration mixture of both GM and non-GM powder materials, which makes it easier to assess the stability of both powders. The short-term stability study was carried out using an isochronous design [15]. In this approach, samples of ERM-BF410gn were stored for a defined 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-BF410n is a dried soya bean seed powder, which has been prepared in a similar manner to previous GMO CRM soya powders produced by IRMM and which have similar water content and particle size distribution. Therefore, the data obtained from the stability monitoring of previous soya bean GMO CRMs were used to assess the long-term stability of ERM-BF410n, and to estimate the uncertainty associated with storage of this CRM.

5.1 Short-term stability study

For the short-term stability study, units of ERM-BF410gn were stored at 4 °C, 18 °C and 60 °C for each of 1, 2 and 3 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 GTS 40-3-2 soya

bean mass fraction due to the simulated shipping conditions. The slopes of the regression lines were tested for statistical significance. There were no trends that were 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 D.

5.2 Long-term stability study

Data from the stability monitoring program for GMO CRMs were available. Previously released soya bean powder CRMs were analysed for their GM mass fraction with 23 data points over a period of 8 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 soya bean GMO CRMs produced and stored in the same way as ERM-BF410n, can be used to estimate the stability uncertainty contribution for ERM-BF410n, 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 E.

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:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum\left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 4}$$

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

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 soya bean 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 6.

Table 6: 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-BF410n	0.7	1.3

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 two candidate CRMs, under the label ERM-BF410n, are gravimetrically diluted mixtures of the pure non-GM and GM soya bean seed powders. ERM-BF410n is certified for the mass fraction of GTS 40-3-2 soya bean event. Gravimetric mixing was the method of choice based on a primary method of measurement confirmed by PCR analysis.

6.1 Genetic purity of the materials

The genetic purity with respect to the GTS 40-3-2 soya bean event of the GM and non-GM batches used for the processing of the candidate CRMs was investigated to calculate the certified value. The values were taken from the previous productions as the same raw materials were used.

For the purity of the GM raw material the genetic identity of randomly selected seeds has been checked during the previous production. No indication was found that the GM soya bean material contained seeds that were negative for the event GTS 40-3-2 soya bean (Section 3.1). Based on a statistical analysis of the distribution of the probability to find a negative seed in the GM raw material, it could be concluded that the purity was higher than 94 % (95 % confidence level). For the calculation of the certified value, a GM purity of the seed batch of 100 % was used, based on the actual number of positive seeds detected per

total number of seeds analysed (46 plants were grown out of 46 seeds, and from each plant 50 mg of leaf tissue were used for the analyses).

The genetic purity of the non-GM starting material was presented via the LOD of the qPCR method performed during the certification of ERM-BF410ak. The study showed that the non-GM powder used for the mixtures (ERM-BF410dn and ERM-BF410gn), did not contain traces of GTS 40-3-2 soya bean above the LOD of the qPCR method used (Sections 3.1 and 3.4).

Since no evidence of contamination was found in the non-GM and the GM materials, 100 % genetic purity was used for the calculation of the certified mass fraction of GTS 40-3-2 soya bean in the powder mixtures. The statistically established genetic purity of at least 93.5 % (Section 3.1) 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 GTS 40-3-2 soya bean event. The values were calculated according to the following equations:

$$\text{GM mass fraction [g/kg]} = \frac{m_{GM,dry}}{m_{GM,dry} + m_{nonGM,dry}} \times 1000 \qquad \textbf{Equation 6}$$

$$m_{GM,dry} = m_{GM} \times (1 - WMF_{GM}) \qquad \textbf{Equation 7}$$

$$m_{nonGM,dry} = m_{nonGM} \times (1 - WMF_{nonGM}) \qquad \textbf{Equation 8}$$

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

The data supporting the calculation of the mass fractions of GTS 40-3-2 soya bean are summarised in Table 7.

Table 7: Subsequent mixing of pure GTS 40-3-2 GM soya bean seed powder with pure non-GM powder to prepare the ERM-BF410dn and ERM-BF410gn materials

Candidate CRM	GM powder [1]			Non-GM powder [1]		Mixtures
	GM 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-BF410gn	1000.0 [2]	6.8 ± 0.03	273.67	11.5 ± 0.7	2476.05	100
ERM-BF410dn	100.0 [3]	11.2 ± 0.7	249.96	11.5 ± 0.7	2250.21	10.0

[1] Calculations of the mass fraction of GTS 40-3-2 soya bean in the powder mixtures are based on a 100 % genetic purity with regard to GTS 40-3-2 soya bean of the non-GM and GM powder materials.

[2] Pure GTS 40-3-2 GM soya bean seed powder was used for the preparation of ERM-BF410gn.

[3] GM powder mixture ERM-BF410gn was used for the preparation of ERM-BF410dn.

The uncertainties of the certified GTS 40-3-2 soya bean mass fractions (u_{char}) have several components, i.e. the uncertainty arising from weighing ($u_{char,1}$), the uncertainty of the determination of the water mass fraction ($u_{char,2}$), and the uncertainties associated with the determination of the genetic purity concerning the GTS 40-3-2 soya bean event of the non-GM and GM powder materials ($u_{char,3}$ and $u_{char,4}$, respectively). Based on a statistical analysis of the probability distribution of finding a negative seed in the GM raw material, it was concluded that the genetic purity of the event GTS 40-3-2 soya bean event in this CRM, was higher than 93.5 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value (Table 8).

Table 8: Uncertainty budgets for the mass fractions of GTS 40-3-2 soya bean in ERM-BF410n

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-BF410dn	10	0.0179	0.0065	0.2021	0.1879	0.2766
ERM-BF410gn	100	0.1206	0.0528	0.2021	1.8786	1.8941

[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 powder material (LOD = 0.7 g/kg), based on the half-width of the interval between 0 and 0.7 g/kg, divided by the square root of 3 (rectangular distribution), performed for the certification of ERM-BF410k.

[4)] Standard uncertainty of the genetic purity estimation of the GM raw material (> 93.5 %), based on the interval between 93.5 % and 100 % divided by the square root of 3 (rectangular distribution).

6.3 Consistency measurements

Real-time PCR measurements confirmed that no mixing errors were made during the preparation of the candidate CRMs (Section 3.4). Additionally, gel electrophoresis proved that the DNA was not degraded during the 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 7).

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:

$$U_{CRM,rel} = k \cdot \sqrt{u_{char,rel}^2 + u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2} \qquad \textbf{Equation 9}$$

- 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 two 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-BF410dn and ERM-BF410gn.

The certified values and their uncertainties are summarised in Table 9.

Table 9: Certified values and their uncertainties for ERM-BF410n

CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{lts} [g/kg]	U_{CRM} [1)] [g/kg]
ERM-BF410dn	10.0	0.2766	0.3498	0.0700	0.1299	1.0
ERM-BF410gn	100	1.8941	1.9990	0.6997	1.2994	7

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

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The certified identity is based on the documentary traceability to the GTS 40-3-2 soya bean event, (Biosafety Clearing house, record ID 14796) [8].

Quantity value

The traceability chain for ERM-BF410dn and ERM-BF410gn is based on the use of calibrated balances and a thorough control of the weighing procedure.

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

8.2 Commutability

ERM-BF410n was prepared gravimetrically from non-GM and GM seed powders with the aim to resemble as much as possible the kind of thresholds set in the corresponding EU legislation for food and feed, namely mass fractions.

ERM-BF410n is intended to be used as calibrant for qPCR measurements of the soya bean GM event GTS 40-3-2 in food and feed. Consequently, this certified reference material is establishing, together with the measurement method validated by the EU-RL GMFF [10], the arbitrary reference system required for quantification of GTS 40-3-2 soya bean. Therefore, commutability, which is a crucial characteristic for reference materials in case that different measurement methods would be applied, does not have to be considered here.

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. As it is a milled material, 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 soya bean powder.

9.4 Use of the certified value

The main purpose of these materials is for calibration or quality control of GTS 40-3-2 soya bean 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 GTS 40-3-2 soya bean 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-BF410n 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 [18]).

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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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] Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired. Off. J. Eur. Union, L 166:9-15

[7] 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

[8] Soya event MON-04032-6 (http://bch.cbd.int/database/record.shtml?documentid=14796) Biosafety clearing house ID 14796

[9] S. Trapmann, D. Gancberg, P. Corbisier, S. Mazoua, A. Merveillie, M. F. Tumba, H. Emons. Certification of reference materials of soya seed powder with different mass fractions of genetically modified Roundup Ready® soya ERM®-BF410k. EUR report 23504 - European Community Luxembourg - 2008, ISBN 978-92-79-09797-3

[10] European Reference Laboratory for GM food and feed (EU-RL GMFF) Status of dossiers, http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx (last update on 25/01/2016)

[11] TaqMan® Universal PCR Master Mix protocol, Applied Biosystems, http://docs.appliedbiosystems.com/pebiodocs/04304449.pdf (last accessed on 27/10/2014)

[12] 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

[13] 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

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

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

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

[17] Vesper H., Emons H., Gnezda M., Jain C.P., Miller W.G., Rej R., Schumann G., Tate J., Thienpont L., Vaks J.E. (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] Linsinger T.P.J., ERM Application Note 1: Comparison of a measurement result with the certified value, http://www.erm-crm.org (last accessed on 19/12/2014)

Annexes

Annex A: CTAB-tip20 DNA extraction method (as modified in-house)

Solutions and reagents

1. CTAB buffer A 1%
   - 1 % (w/v) CTAB
   - 0.7 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. 2-mercaptoethanol
4. Chloroform-Octanol (24:1)
5. 1.2 M NaCl
6. 1 mM Tris, pH 8.0
7. 0.01 mM Na_2EDTA, pH 8.0
8. Proteinase K, 20 mg/mL
9. RNase A, 100 mg/mL
10. Qiagen Genomic-tip20 columns
11. Qiagen QBT equlibration buffer
12. Qiagen QC washing buffer
13. Qiagen QF elution buffer
14. Ispropanol
15. Ethanol
16. 70 % (v/v) Ethanol
17. TE low buffer

DNA extraction protocol

a) Weigh 200 mg soya bean powder into a 2 mL microcentrifuge tube
b) Add 1.3 mL of CTAB Buffer A 1% + 5 µL RNase A + 6.5 µL Proteinase K + 26 µL 2-mercaptoethanol and mix by vortexing
c) Incubate 1 h at 65 °C, shaking at 1400 rpm
d) Centrifuge for 10 min at 16000 x *g* at RT
e) Transfer 750 µL of supernatant to a 2 mL microcentrifuge tube containing 1 mL of chloroform:octanol (24:1)
f) Mix thoroughly by inverting, incubate 5 min at RT
g) Centrifuge for 10 min at 16000 x *g* at RT
h) Transfer 600 µL of supernatant to a new 2 mL microcentrifuge tube containing 700 µL of CTAB Buffer B
i) Mix thoroughly by inverting, incubate 30 min at RT
j) Centrifuge for 20 min at 16000 x *g* at RT
k) Discard the supernatant by pipetting and conserve the pellet
l) Add 200 µL of 1.2 M NaCl
m) Incubate 5 min at 50 °C, shaking at 1400 rpm
n) Add 1.6 mL of G2 buffer + 2.5 µL of RNase A + 20 µL of Proteinase K
o) Incubate 1 h at 50 °C, shaking at 500 rpm
p) Centrifuge 5 min at 16000 x *g* at RT
q) Equilibrate a Qiagen Genomic-tip 20/G column with 1 mL of QBT buffer
r) Apply the sample to the equilibrated Genomic-tip 20/G column by pipetting
s) Wash the genomic-tip 20/G column with 3 mL of QC buffer
t) Elute the genomic DNA with 1 mL of QF buffer (pre-warmed at 50 °C) and collect the DNA in a 2 mL tube
u) Add 700 µL of isopropanol to each tube, invert 10 times
v) Centrifuge for 30 min at 10000 x *g* at 4 °C, discard the supernatant by pipetting
w) Wash the pellet with 1 mL of 70 % ethanol
x) Centrifuge 10 min at 13000 x *g* at 4 °C
y) Discard the supernatant by pipetting and air-dry the pellet for 10 min
z) Dissolve the DNA pellet in 80 µL of TE Low buffer preheated at 50 ºC, incubate 10 min at 50 °C while shaking at 500 rpm. Let the pellet to dissolve completely overnight at RT and store the samples at + 4 °C (short term) or at -20 °C (long term).

Annex B: Results of the comparison measurements of ERM-BF410n with the previous batch (ERM-BF410k)

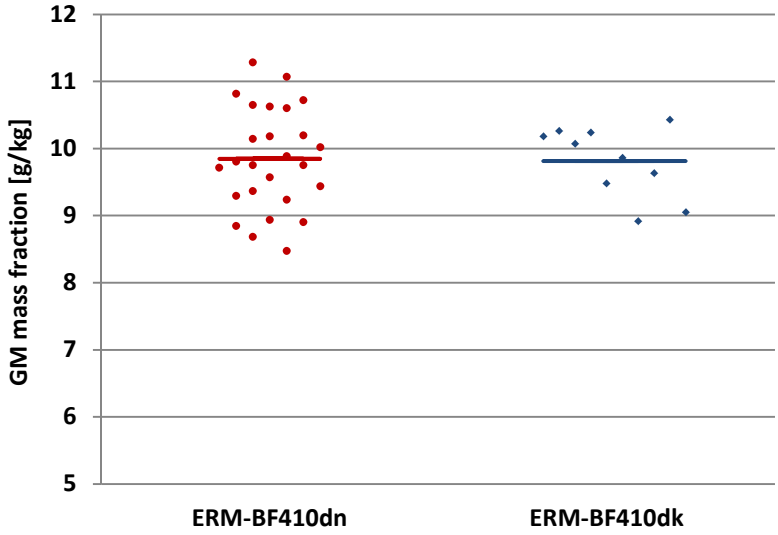

Figure B1: Real-time PCR measurement results with the mean values for ERM-BF410dn and ERM-BF410dk. Two samples (extraction replicates) were measured from each of 13 randomly selected bottles (N = 13, n = 2) for ERM-BF410dn and 5 selected bottles (N = 5, n = 2) from ERM-BF410dk. Each of the samples was measured in three real-time PCR replicates under intermediate precision conditions.

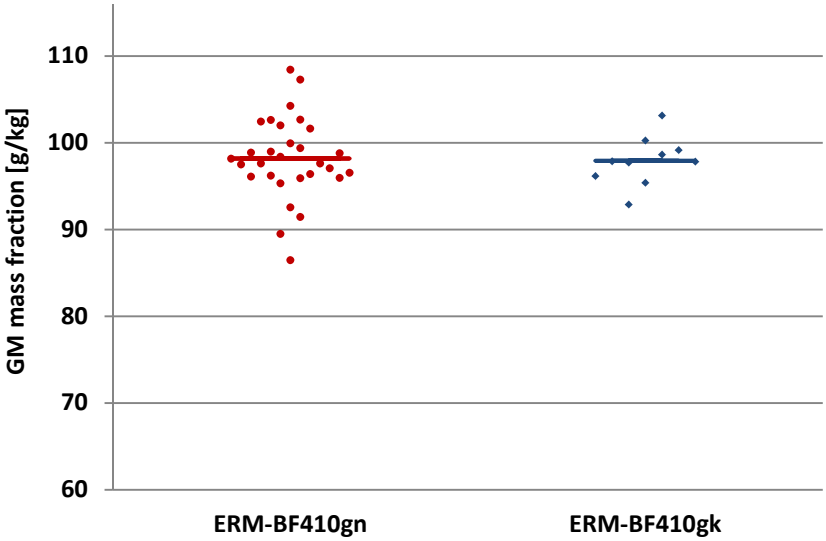

Figure B2: Real-time PCR measurement results with the mean values for ERM-BF410gn and ERM-BF410gk. Two samples (extraction replicates) were measured from each of 15 randomly selected bottles (N = 15, n = 2) for ERM-BF410gn and 5 selected bottles (N = 5, n = 2) from ERM-BF410gk. Each of the samples was measured in three real-time PCR replicates under intermediate precision conditions.

Annex C: Results of the homogeneity measurements

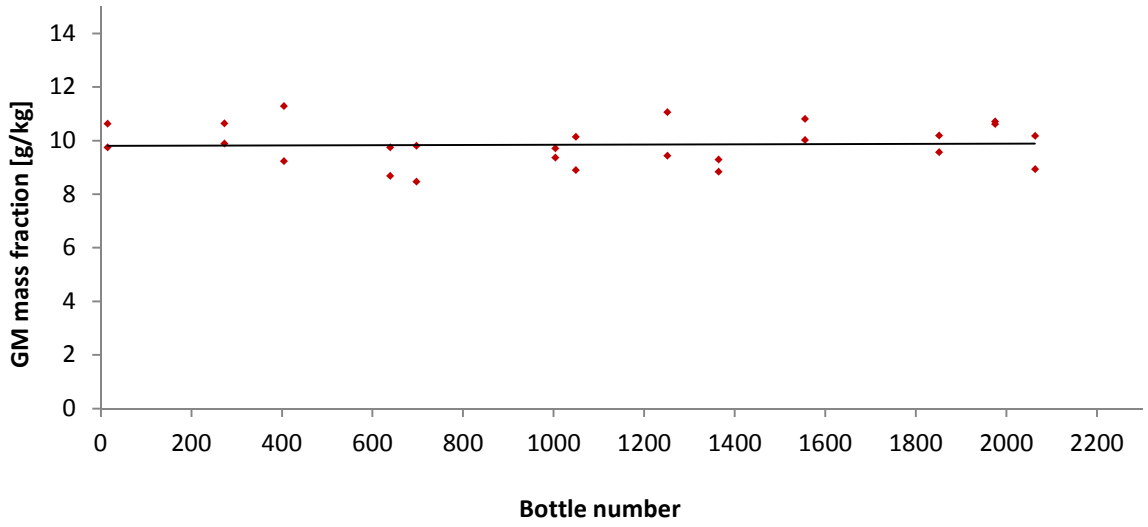

Figure C1: Real-time PCR measurement results for ERM-BF410nd. Two samples (extraction replicates) were measured from each of 13 randomly selected bottles (N = 13, n = 2), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

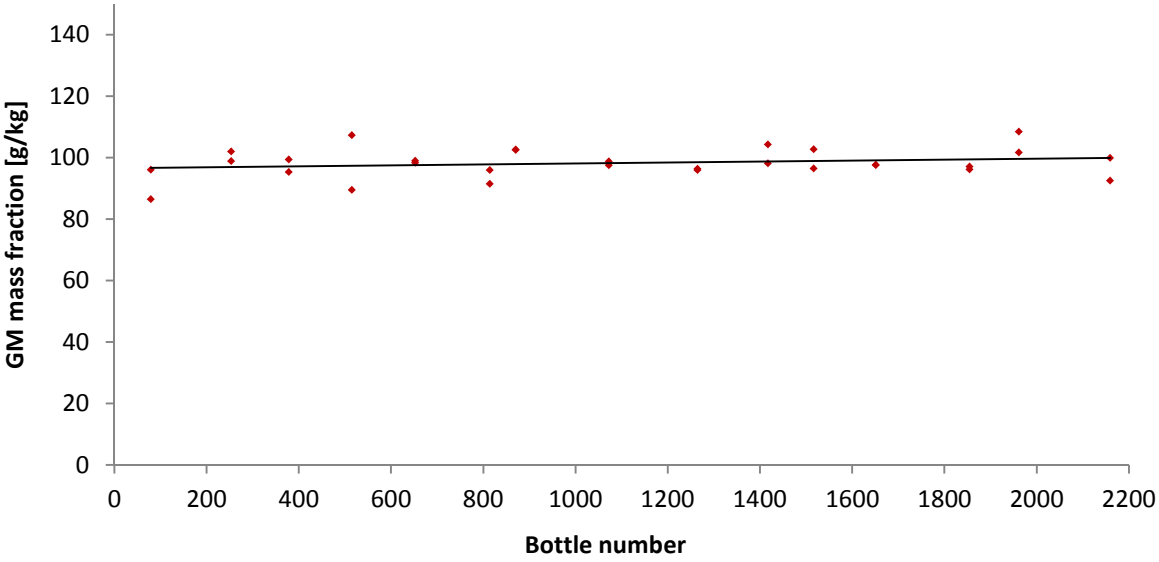

Figure C2: Real-time PCR measurement results for ERM-BF410gn. Two samples (extraction replicates) were measured from each of 15 randomly selected bottles (N = 15, n = 2), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

Annex D: Results of the short-term stability measurements

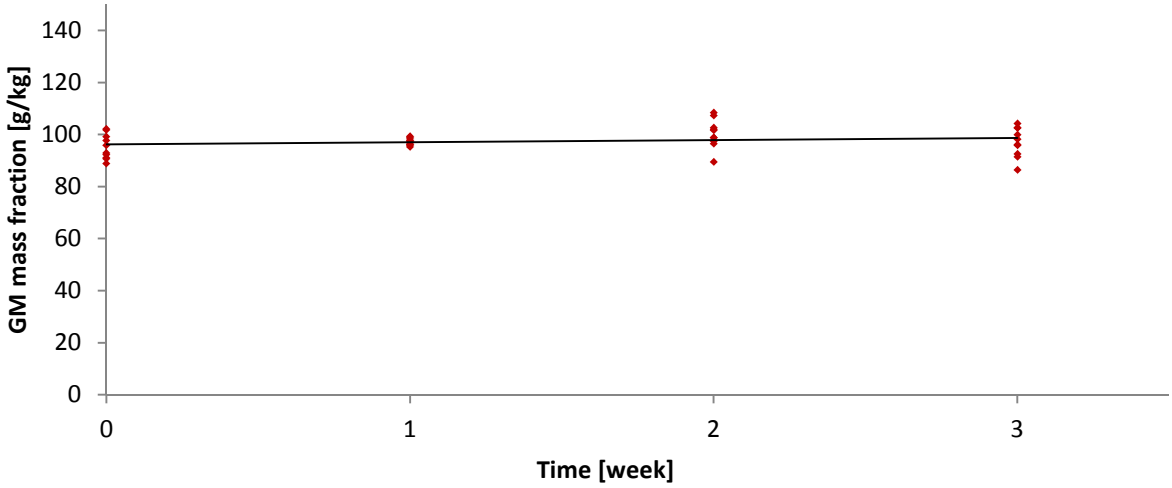

Figure D1: Real-time PCR measurement results for ERM-BF410gn during short-term stability testing at 4 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

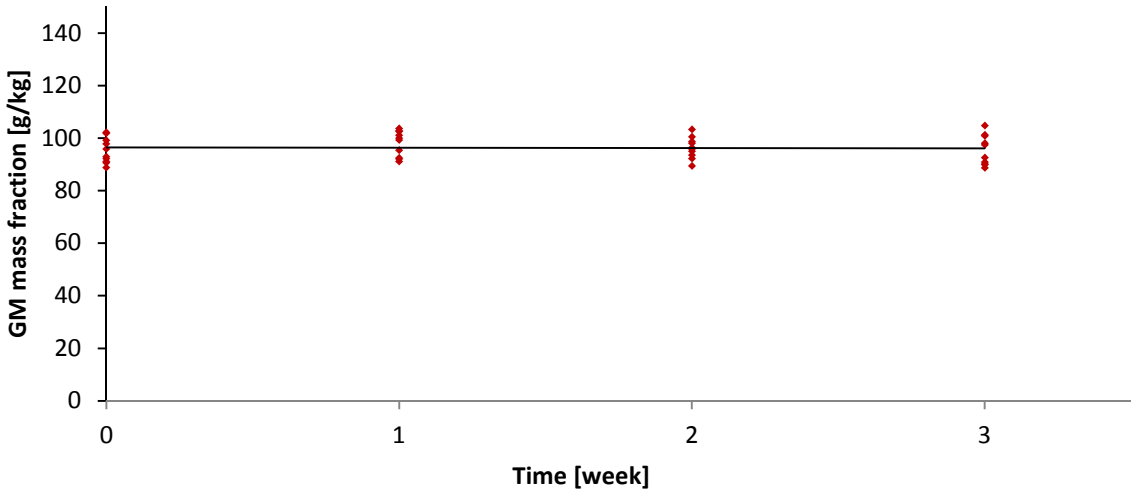

Figure D2: Real-time PCR measurement results for ERM-BF410gn during short-term stability testing at 18 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

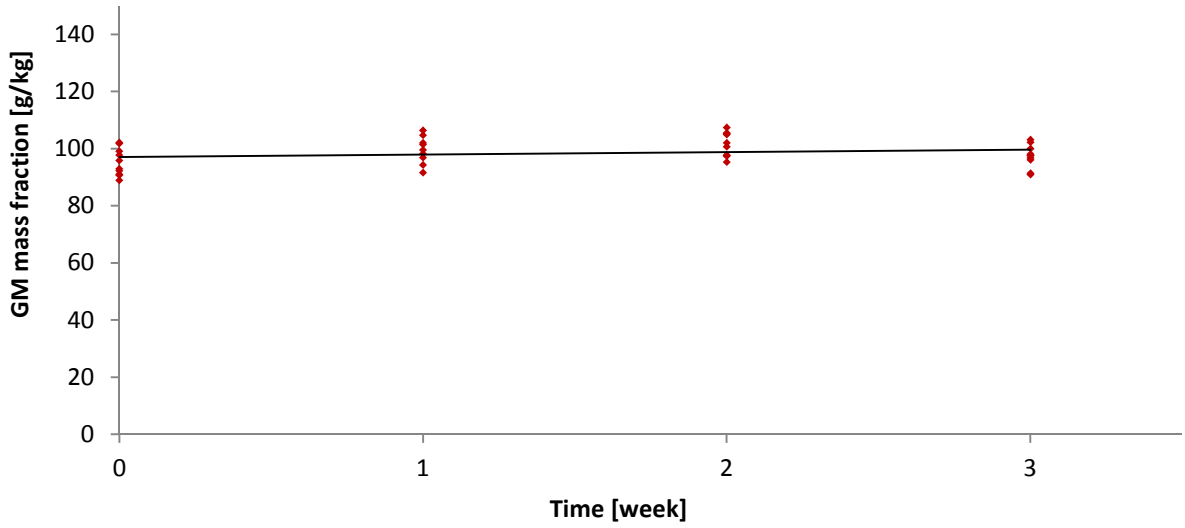

Figure D3: Real-time PCR measurement results for ERM-BF410gn during short-term stability testing at 60 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

Annex E: Results of the long-term stability measurements

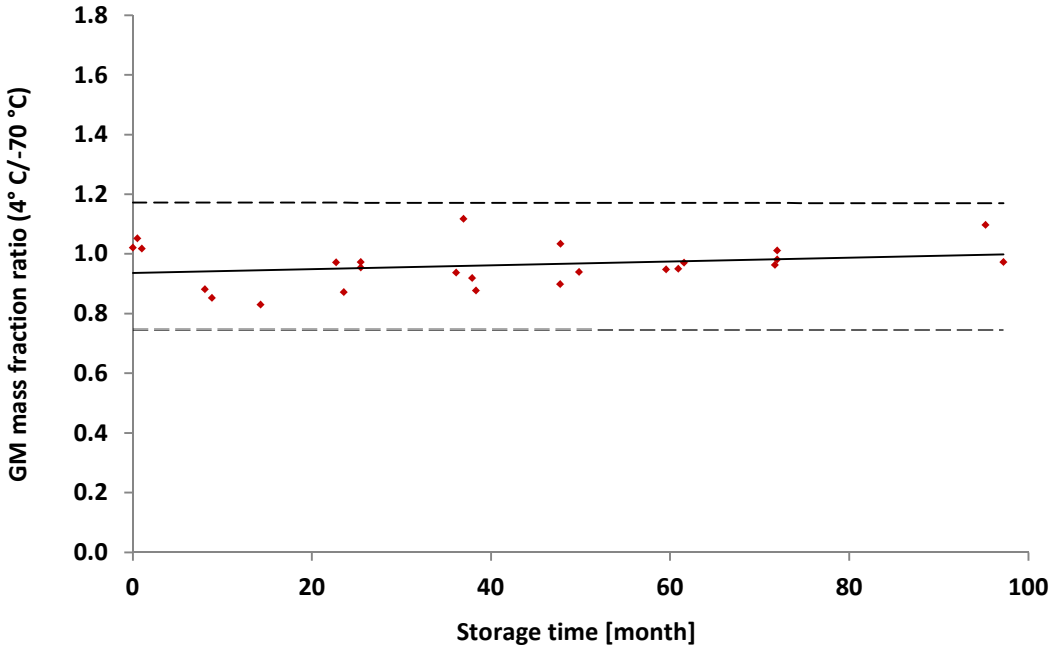

Figure E1: Real-time PCR measurement results of ERM-BF410n (1, 2 and 3 weeks) compared to ERM-BF410k, ERM-BF425, ERM-BF426, ERM-BF432 and ERM-BF437 (data from the post-certification monitoring). The dashed lines give the limits of 3 *s* obtained for the measurement results. The straight line is a least-squares linear regression for all data points.

European Commission

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Certified Reference Materials ERM®-BF410dn and ERM®-BF410gn

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