# CERTIFICATION REPORT

# The certification of different mass fractions of GMB151 in soya bean powder:

# Certified Reference Materials
# ERM®-BF443a, ERM®-BF443b, ERM®-BF443c, ERM® BF443d and ERM®-BF443e

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Abstract

This report describes the production of ERM®-BF443, which are soya bean powder materials certified for the mass fraction of GMB151 (unique identifier BCS-GM151-6). These materials were produced and certified in accordance with ISO 17034:2016 [ ] and ISO Guide 35:2017. ERM BF443 was produced within the scope of ISO 17034 accreditation.
Genetically modified (GM) soya bean seeds of the GMB151 event and seeds from a non-GM soya bean variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM soya bean seed powder were prepared gravimetrically.
The certified reference materials (CRMs) are available in glass vials containing at least 1 g of dried soya bean powder, which were sealed under an atmosphere of argon.
Between-unit homogeneity was quantified and stability during dispatch and storage was assessed in accordance with ISO Guide 35:2017 The minimum sample size for one measurement is 200 mg.
The certified value was obtained from the gravimetric preparations, taking into account the genetic purity of the base materials with respect to the GMB151 soya bean and their water mass fractions. The certified values were confirmed by event-specific real-time polymerase chain reaction (PCR) (measurements produced by a laboratory with demonstrated competence and adhering to ISO/IEC 17025:2005).
Uncertainties of the certified values were calculated in accordance with ISO 17034:2016 and ISO Guide 35:2017 and include uncertainties related to possible inhomogeneity, instability and characterisation.
The materials are intended for the calibration or quality control of quantitative PCR measurements to identify GMB151 soya bean and quantify its mass fraction. As with any reference material, they can be used for establishing control charts or validation studies.
Before release of the CRMs, the certification project was subjected to internal peer-review.

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# The certification of different mass fractions of GMB151 in soya bean powder:

# Certified Reference Materials ERM®-BF443a, ERM®-BF443b, ERM®-BF443c, ERM® BF443d and ERM®-BF443e

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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 ERM®-BF443, which are soya bean powder materials certified for the mass fraction of GMB151 (unique identifier BCS-GM151-6). These materials were produced and certified in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2]. ERM-BF443 was produced within the scope of ISO 17034 accreditation.

Genetically modified (GM) soya bean seeds of the GMB151 event and seeds from a non-GM soya bean variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM soya bean seed powder were prepared gravimetrically.

The certified reference materials (CRMs) are available in glass vials containing at least 1 g of dried soya bean powder, which were sealed under an atmosphere of argon.

Between-unit homogeneity was quantified and stability during dispatch and storage was assessed in accordance with ISO Guide 35:2017 [2]. The minimum sample size for one measurement is 200 mg.

The certified value was obtained from the gravimetric preparations, taking into account the genetic purity of the base materials with respect to the GMB151 soya bean and their water mass fractions. The certified values were confirmed by event-specific real-time polymerase chain reaction (PCR) (measurements produced by a laboratory with demonstrated competence and adhering to ISO/IEC 17025:2005 [3]).

Uncertainties of the certified values were calculated in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The materials are intended for the calibration or quality control of quantitative PCR measurements to identify GMB151 soya bean and quantify its mass fraction. As with any reference material, they can be used for establishing control charts or validation studies.

Before release of the CRMs, the certification project was subjected to internal peer-review.

The following values were assigned:

| GMB151 soya bean mass fraction [1] | | |
|---|---|---|
| | Certified value [2] [g/kg] | Uncertainty [g/kg] |
| ERM-BF443a | 0 [3] | + 0.04 [4]<br>- 0 |
| ERM-BF443b | 1000 [5] | + 0 [6]<br>- 10 |
| ERM-BF443c | 1.00 [7] | 0.09 [8] |
| ERM-BF443d | 10.0 [7] | 0.8 [8] |
| ERM-BF443e | 100 [7] | 6 [8] |

[1] Genetically modified soya bean with the unique identifier BCS-GM151-6.

[2] Certified values are values that fulfil the highest standards of accuracy. The certified values and its uncertainty are traceable to the International System of Units (SI).

[3] The certified reference material has been produced from conventional, non-modified soya bean seeds. No contamination was detected in this material when using an event-specific quantitative polymerase chain reaction assay targeting the GMB151 soya bean event. The limit of detection (LOD) of the PCR method was 0.04 g/kg (LOD = 3.3 * $s$, and $s$ used from lowest concentration with $s_{rel} \leq 25$ %). With 95 % confidence, the true GMB151 soya bean mass fraction of the material is below 0.04 g/kg.

[4] The asymmetric uncertainty is based on the 95 % confidence interval of the LOD. With 95 % confidence, the true GMB151 mass fraction of the material is therefore between 0 and 0.04 g/kg. If using ERM BF443a for calibration the value 0 g/kg with an expanded uncertainty of + 0.04 / - 0 g/kg should be used. The corresponding standard uncertainty is equal to 0.04 g/kg / √3 = 0.03 g/kg.

[5] This certified reference material was produced from genetically modified GMB151 soya bean seeds. The certified value is based on the genetic purity of the soya bean seeds, > 99 % with regard to GMB151 soya bean. With 95 % confidence, the true GMB151 soya bean mass fraction of the material is above 990 g/kg.

[6] The asymmetric uncertainty is based on the 95 % confidence interval of the purity data from BASF Belgium Coordination Center (Gent, BE). With 95 % confidence, the true GMB151 mass fraction of the material is therefore between 990 and 1000 g/kg. If using ERM BF443b for calibration the value 1000 g/kg with an expanded uncertainty of + 0 / - 10 g/kg should be used. The corresponding standard uncertainty is equal to 10 g/kg / √3 = 6 g/kg.

[7] Certified values based on the masses of dried genetically modified GMB151 soya bean powder and dried non-modified soya bean powder that were mixed, taking into account their respective genetic purity with regard to GMB151 soya bean and their respective water content.

[8] The uncertainty of the certified value is the expanded uncertainty with a coverage factor $k$ = 2 corresponding to a level of confidence of 95 %, estimated in accordance with ISO 17034:2016 and ISO Guide 35:2017.

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# Glossary

| | |
|---|---|
| ANOVA | Analysis of variance |
| BBCC | BASF Belgium Coordination Center |
| CI | Confidence interval |
| Cq | Quantification cycle (also referred to as threshold cycle, Ct) |
| CRM | Certified reference material |
| CTAB | Cetyltrimethylammonium bromide |
| DNA | Deoxyribonucleic acid |
| EC | European Commission |
| EDTA | Ethylenediaminetetraacetic acid |
| ERM® | Trademark owned by the European Commission; used by the JRC for reference materials |
| EU | European Union |
| EURL-GMFF | European Union Reference Laboratory for Genetically Modified Food and Feed |
| GM | Genetically modified |
| GMO | Genetically modified organism |
| GUM | Guide to the Expression of Uncertainty in Measurement |
| h | hour |
| ISO | International Organization for Standardization |
| JRC | Joint Research Centre of the European Commission |
| *k* | Coverage factor |
| KFT | Karl Fischer titration |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| $MS_{between}$ | Mean of squares between-unit from an ANOVA |
| $MS_{within}$ | Mean of squares within-unit from an ANOVA |
| *n* | Number of replicate analysis per unit |
| *N* | Number of units analysed |
| n.a. | Not applicable |
| n.c. | Not calculated |
| PCR | Polymerase chain reaction |
| PSA | Particle size analysis |
| rel | Index denoting relative figures (uncertainties etc.) |
| RM | Reference material |

| | |
|---|---|
| *RSD* | Relative standard deviation |
| *RSE* | Relative standard error (=*RSD*/$\sqrt{n}$) |
| $r^2$ | Coefficient of determination of the linear regression |
| *s* | Standard deviation |
| $s_{bb}$ | Between-unit standard deviation; an additional index "rel" is added when appropriate; this parameter is linked to the homogeneity of the material |
| $s_{between}$ | Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate |
| SI | International System of Units |
| $s_{wb}$ | Within-unit standard deviation; this parameter is linked to the homogeneity of the material |
| $s_{within}$ | Standard deviation within groups as obtained from ANOVA; 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 | Buffer containing TRIS and EDTA |
| $t_{sl}$ | Proposed shelf life |
| $t_{tt}$ | Proposed transport time |
| TRIS | Tris(hydroxymethyl)aminomethane |
| *u* | Standard uncertainty |
| *U* | Expanded uncertainty |
| $u^*_{bb}$ | Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method intermediate precision; 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 |
| $u_c$ | Combined standard uncertainty; 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 |
| $u_\Delta$ | Combined standard uncertainty of measurement result and certified value |
| $u_{lts}$ | Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate |

| | |
|---|---|
| $u_{meas}$ | Standard measurement uncertainty |
| $U_{meas}$ | Expanded measurement uncertainty |
| $u_{rec}$ | Standard uncertainty related to possible between-unit inhomogeneity modelled as rectangular distribution; an additional index "rel" is added as appropriate |
| $u_{sts}$ | Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate |
| VIM | International Vocabulary of Metrology – Basic and General Concepts and Associated Terms |
| V-KFT | Volumetric Karl Fischer titration |
| $\alpha$ | Significance level |
| $\Delta_{meas}$ | Absolute difference between mean measured value and the certified value |
| $\nu_{s,meas}$ | Degrees of freedom for the determination of the standard deviation $s_{meas}$ |
| $\nu_{MSwithin}$ | Degrees of freedom of $MS_{within}$ |
| $X_{10}$ | Particle diameter corresponding to 10 % of the cumulative undersize distribution (here by volume) |
| $X_{50}$ | Median particle diameter corresponding to 50$^{th}$ percentile of the cumulative undersize distribution (here by volume) |
| $X_{90}$ | Particle diameter corresponding to 90 % of the cumulative undersize distribution (here by volume) |
| $\bar{y}$ | Mean of all results of the homogeneity study |

# 1 Introduction

## 1.1 Background

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 [4]. 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 [5]. 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.

BASF Agricultural Solutions Seed LLC (US) is the owner of the genetically modified (GM) GMB151 soya bean event (unique identifier code BCSGM151-6, following Commission Regulation (EC) No 65/2004 [6]). This transgenic crop has been modified for resistance to nematode plant parasites and has herbicide tolerance to 4-hydroxyphenylpyruvate dioxygenase (HPPD) inhibitors. The GMB151 soya bean contains the *Bacillus thuringiensis Cry14Ab1* gene for resistance to nematode plant parasites, such as soya bean cyst nematode. For herbicide tolerance, it has a *Pseudomonas fluorescens HPPD* gene with point mutations in the C-terminal to allow for and enhance tolerance to HPPD-inhibitor herbicides, such as isoxaflutole and mesotrione [7].

In 2019, BASF Belgium Coordination Center (BBCC) (Antwerp, BE) commissioned the European Commission's Joint Research Centre, Directorate F – Health, Consumers to produce a certified reference material (CRM) for the quantification of GMB151 soya bean. The CRM produced by the JRC received the code ERM-BF443 and is composed of five CRMs containing different mass fractions of GMB151 soya bean. Like previous CRM productions, the codes used for the different concentrations of the mass fraction of GMB151 soya bean followed the labelling pattern where the ERM-BF443a and ERM-BF443b are the pure non-GM and GM materials, and ERM-BF443c, d and e are 0.1 %, 1 % and 10 % materials, respectively.

## 1.2 Choice of the material

The set of CRMs ERM-BF443 consists of 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. The nominal mass fraction levels of GMB151 in the soya bean powder for ERM-BF443c and ERM-BF443d were chosen to be close to the 0.1 (m/m) % threshold for feed and the 0.9 % labelling threshold respectively, established by the EU legislation [5, 4]. ERM-BF443b was prepared as calibrant for the quantitative polymerase chain reaction (qPCR) method and ERM-BF443a to serve as a negative control while ERM-BF443e is an additional level for samples at a higher GM levels.

## 1.3 Outline of the CRM project

The production of a CRM as defined in ISO 17034 [1] is a project comprising planning, processing of the material, homogeneity and stability testing, characterisation and assigning of the property values and finally distribution and post-certification monitoring to control stability.

Alongside the pure non-GM material ERM-BF443a and the pure GM material ERM-BF443b, mixtures of non-GM and GM soya bean powder were prepared gravimetrically. The first mixed material ERM-BF443e was prepared by mixing pure GM with non-GM soya bean powder. ERM-BF443d was prepared by further dilution of ERM-BF443e, and ERM-BF443c was prepared by further dilution of ERM-BF443d, in both cases with non-GM soya bean powder. The different mass fractions of ERM-BF443 were certified using a gravimetric approach, the details of which are described in Section 6.2.

The event specific PCR method for GMB151 soya bean was developed by Bayer CropScience (before the acquisition of Bayer's assets by BASF on the 1st of August, 2018) and was validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) (Ispra, IT) in an inter-laboratory international collaborative study both in accordance with EC Regulation [4, 8].

The ERM-BF443 CRMs and the validated method set the reference point for the quantification of GMO food/feed samples.

The genetic purity with respect to the GMB151 soya bean event of the non-GM and GMB151 soya bean seeds was investigated by BBCC (Gent, BE) and was used in the calculations of the certified value and their uncertainties.

Uncertainties of certified values were estimated in compliance with ISO 17034 [1], which implements the basic principles of ISO/IEC Guide 98 (GUM) [9].

The CRM project, including the certification approach and the evaluation of the obtained measurement data, was subjected to peer-review involving internal experts.

# 2 Participants

## 2.1 Project management, processing, characterisation, analytical measurements and data evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Unit F.6 (Reference Materials Unit) (Geel, BE)
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

## 2.2 Provider of raw material and quantification method

BBCC (Gent, BE) provided the raw materials, the non-GM and GM GMB151 soya bean seeds. Bayer CropScience (before the acquisition of Bayer's assets by BASF on the 1st of August, 2018) provided the event specific quantitative PCR method which was validated and published in July 2020 by the EURL-GMFF (Ispra, IT).

## 2.3 Homogeneity, stability and confirmation measurements

Agency for Health and Food Safety (AGES), Department for Molecular Biology and Microbiology (Vienna, AT)
(accredited to ISO/IEC 17025:2005 for GMO quantification, DNA based procedures, Akkreditierung Austria No. 0371)

# 3 Material processing and process control

## 3.1 Origin of the starting material

BBCC (Gent, BE) supplied the Joint Research Centre, Directorate F – Health, Consumers and Reference Materials (JRC, Geel, BE) with non-GM soya bean seeds and GMB151 soya bean seeds to prepare the ERM-BF443 CRMs. According to the information provided by BBCC (Gent, BE) the GMB151 seeds are homozygous. After arrival, the seeds were stored at (4 ± 3) °C in the dark until processing.

The genetic purity with respect to the GMB151 event of the GM soya bean seeds was according to information from BBCC (Gent, BE) higher than 99 % and the genetic impurity lower than 0.01 %, both values with a 95 % confidence level. The half-width of the difference between the measured purity of > 99 % and the 100 % purity 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 GMB151 soya bean event was investigated using the processed seed powder. Five units of ERM-BF443a were randomly selected and the DNA was extracted from two samples (extraction replicates) taken from each unit ($N$ = 5, $n$ = 2). Each DNA extract was then analysed in three replicates by quantitative PCR method, with a limit of detection (LOD) of 0.04 g/kg. This analysis did not detect the GMB151 event (Section 3.5). The LOD of the event-specific quantitative PCR method was taken into consideration when the uncertainty of the certified value of ERM-BF443a was calculated (Section 7). For establishing the LOD, data from eight qPCR GMB151 calibration curves were used and the LOD calculated as 3.3-fold $s$ (with standard deviation $s$ of lowest calibration point with $s_{rel}$ ≤ 25 %).

## 3.2 Processing

For the purpose of this report, the term 'unit' refers to one vial of ERM-BF443a, ERM-BF443b, ERM-BF443c, ERM-BF443d, ERM-BF443e.

One unit of each ERM-BF443a, b, c, d, e, is shown in Figure 1.

**Figure 1:** Set of GMO CRMs ERM-BF443

All soya bean seeds received by the JRC (Geel, BE) were rinsed with water, drained, and dried on trays in the drying chamber of a freeze-dryer at 20 °C for 20 h (Epsilon 2-65D, Martin Christ, Osterode, DE). Approximately 30 kg of non-GM soya bean seeds and 10 kg of GMB151 soya bean seeds were used for the production of the ERM-BF443. The GM and non-GM base materials 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 proven that the solution degraded DNA effectively under the given conditions.
The soya bean seeds were frozen overnight in liquid nitrogen in approximately 5 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. The powder was cold sieved with a 710 µm stainless steel mesh on a sieving machine (Russell Finex, London, UK). After sieving, the coarse fraction (>710 µm) was immediately re-milled. The remaining powder from each base material was cold mixed in a DynaMIX CM200 (WAB, Muttenz, CH) for 1 h to homogenize the distribution of the different types of soya bean seed tissues, since it is known that the milling and sieving processes result in separation of the various seed tissues from each other. After mixing, the powder was maintained at 4 ± 3 °C in an air-tide container. To facilitate homogenous mixing of the powders, the water content of the powders was reduced further by drying them overnight under vacuum in the freeze-dryer at 20 °C and the powders were subsequently mixed for 1 h to homogenise. The final water mass fractions of the non-GM powder and the GM powder were measured as (26.1 ± 1.7) g/kg and (23.0 ± 1.5) g/kg, respectively ($N$ = 1, $n$ = 3), with the expanded uncertainty calculated using a coverage factor $k$ = 2 (Table 1).

The milled raw materials were used to prepare the blank material for GMB151 soya bean (non-GM soya bean seed powder), the pure GM GMB151 soya bean material and three mixtures at nominal mass fraction levels of 1, 10 and 100 g/kg of the GMB151 soya bean 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 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, Göttingen, DE) with an intermediate precision, determined during calibration and expressed as standard uncertainty ($u$), of 0.02 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, while a correction for their respective water content was applied. Portions of the powder materials were weighed into a container and mixed for 1 h by using a Dyna-MIX CM 200 (WAB, Muttenz, CH). The material with a nominal GMB151 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 GMB151 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 and the material with a nominal mass fraction of 1 g/kg was 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 GMB151 soya bean mass fraction for the three powder mixtures (Section 6.2). A feeder, FD – SPAc 4A (MCPI, Meythet, France), was used to fill 10 mL amber glass vials with approximately 1.1 g of powder. To avoid cross contamination the equipment was cleaned between two mass fraction levels and the first 30 units 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 placed in the vials necks. The units were then placed in a freeze-dryer (Epsilon 2-65D, 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 was executed using an HV 100 B 10 semi-automatic capping machine (Bausch & Ströbel, Ilshofen, DE) and a labelling machine (BBK, Beerfelden, DE). Colour-coded caps were used to facilitate the identification of the different mass fraction levels of GMB151 soya bean event:

nominal 0 g/kg = silver (BF443a), nominal 1000 g/kg = black (BF443b), nominal 1 g/kg = gold (BF443c), nominal 10 g/kg = red (BF443d), nominal 100 g/kg = brown (BF443e), consistent with the cap colours of previous JRC CRMs for GMOs. Each of the units 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 units for future analysis according to a random stratified sampling scheme, the remaining units were stored in the dark at 4 ± 3 °C.

## 3.3 Process control

The sample size per unit of ERM-BF443 is 1 g.

Particle size distribution measurements based on laser diffraction (Helos KR, Sympatec GmbH, Clausthal-Zellerfeld, DE) took place during and after final processing of ERM-BF443.

The particle size distributions of both powders were compared. The cumulative volume distribution of the particles derived from laser diffraction data is based on their equivalent spherical diameters, i.e. the diameter of the particles derived from the volume occupied upon their rotation. Based on that the mean diameter of the non-GM and GM base powders was 153.3 µm ± 16.1 µm (*U*) and 149.6 µm ± 8.2 µm (*U*), respectively (Table 1).

However, since most particles are not spherical, the calculated volumes of the particles based on their equivalent diameters will overestimate the mean particle size. Therefore, a three-point specification of the particle size distribution (*N* = 1, *n* = 5) was calculated, consisting of the equivalent sphere diameters at 10 %, 50 % and 90 % of the cumulative volume distribution (Table 1). These size classes were denoted $X_{10}$, $X_{50}$ and $X_{90}$, respectively. A *t*-test showed with 95 % confidence that there were no significant differences between the $X_{10}$, $X_{50}$, $X_{90}$ particle sizes and between the volume mean diameters of the non-GM and GM soya bean powders. Based on the particle volume distributions the non-GM and GM base powders were found to be sufficiently similar not to introduce a DNA extraction bias.

**Table 1:** The water mass fraction determined by Volumetric Karl Fischer titration (V-KFT) and the volume mean diameter and percentiles of cumulative particle size distributions of the base materials by laser diffraction

| Base material | Water mass fraction [g/kg] | | Volume mean diameter [µm] | | $X_{10}$ [µm] | | $X_{50}$ [µm] | | $X_{90}$ [µm] | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\bar{x}$ | *U* | $\bar{x}$ | *U* | $\bar{x}$ | *U* | $\bar{x}$ | *U* | $\bar{x}$ | *U* |
| Non-GM powder | 26.1 [1)] | 1.7 | 153.3 [2)] | 16.1 | 22.9 [3)] | 4.7 | 127.4 [3)] | 21.2 | 320.0 [3)] | 65.5 |
| GM powder | 23.0 [1)] | 1.5 | 149.6 [2)] | 8.2 | 19.9 [3)] | 4.1 | 125.8 [3)] | 20.9 | 315.3 [3)] | 64.3 |

[1)] Mean of one unit (*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.
[2)] Mean of one unit (*N* = 1, *n* = 5). The associated expanded uncertainty (*U*) based on the standard deviation of measurements with a coverage factor of *k* = 2.
[3)] Mean of one unit (*N* = 1, *n* = 5). The associated expanded uncertainty (*U*) with a coverage factor of *k* = 2 has been estimated during validation of the laser diffraction method.

The particle size distribution of the CRMs was determined based on the laser diffraction pattern of the powders. Five randomly selected units from each of the CRMs were analysed in duplicate (*N* = 5, *n* = 2). The equivalent diameters of all particles were below 1020 µm (Figure 2). The volume mean diameters measured by laser diffraction were 155.9 µm ± 9.9 µm (*U*), 156.8 µm ± 9.3 µm (*U*),

160.7 µm ± 13.8 µm (*U*), 160.1 µm ± 12.5 µm (*U*), and 156.3 µm ± 6.1 µm (*U*) for ERM-BF443a, b, c, d and e, respectively.

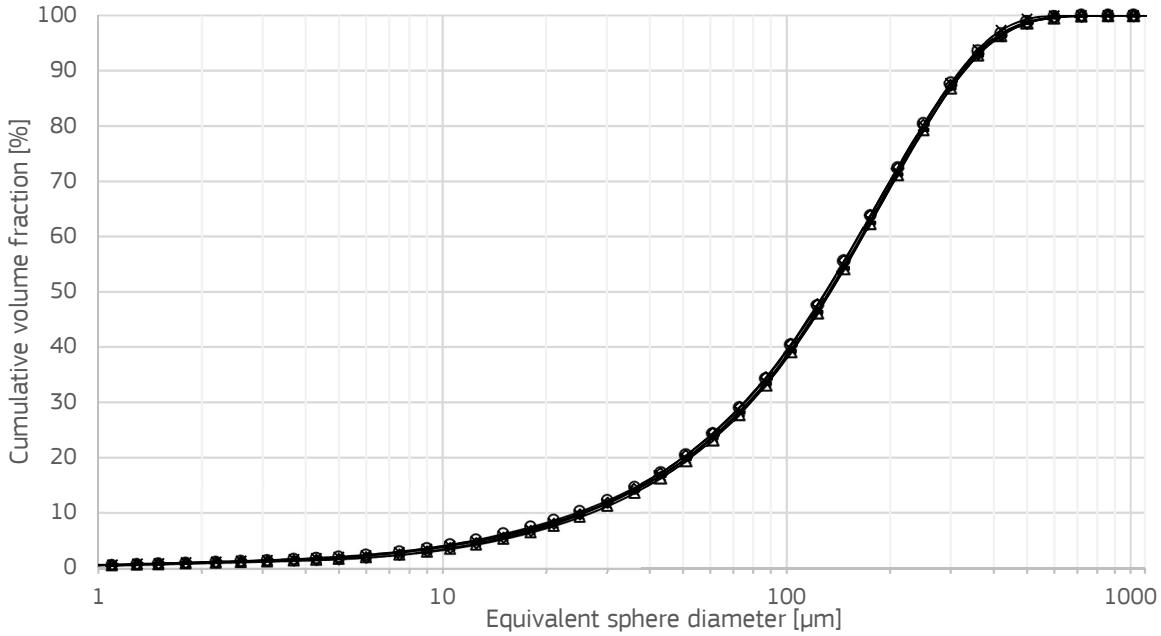


**Figure 2:** Volume-based cumulative distribution of particle size in ERM-BF443a (○), ERM-BF443b (◇), ERM-BF443c (△), ERM-BF443d (-) and ERM-BF443e (x) analysed by laser diffraction (*N* = 5, *n* = 2).

Ten randomly selected units from each CRM were measured by V-KFT to determine the residual mass fraction of water in the powder. The results are summarised in Table 2.

**Table 2:** Water mass fractions of candidate ERM-BF443 CRMs determined by V-KFT (*N* = 10, *n* = 1). The associated expanded uncertainty (*U*) has been estimated during validation of the V-KFT method on soya bean powder

| CRM | Water mass fraction [g/kg] | |
|---|---|---|
| | $\overline{x}$ | *U* (*k* = 2) |
| ERM-BF443a | 26.5 | 1.6 |
| ERM-BF443b | 21.7 | 1.3 |
| ERM-BF443c | 30.2 | 1.8 |
| ERM-BF443d | 21.6 | 1.3 |
| ERM-BF443e | 24.0 | 1.4 |

## 3.4 Total DNA content of the powder materials

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

production of ERM-BF443 contain the same mass of DNA, a slight modification of the classical fractionation method developed initially by Ogur and Rosen [10] 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 [10, 11].
The ratio of the DNA mass extractable from 100 mg of GM and non-GM soya bean powder was found to be (0.994 ± 0.036) ($N$ = 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 soya bean powders using the modified Ogur and Rosen method.

It has to be understood that ERM-BF443 has been developed to set a common reference point for the implementation of EU legislation on GMO thresholds and labelling [4, 5]. The assigned certified GM mass values of the prepared mixtures can only be reproduced by quantitative PCR, if no difference in DNA extractability of GM and non-GM soya bean is imposed by the DNA extraction method selected. While a difference was measured in the size of the non-GM and GM seeds (data not given), no difference in particle size was measured for the non-GM and GM powders (Section 3.3).

## 3.5 Confirmation measurements

As a control for the gravimetric preparations, the mass fraction of GMB151 soya bean event in the mixed materials ERM-BF443c, ERM-BF443d and ERM-BF443e was measured using the event-specific PCR method validated by the EURL-GMFF (Ispra, IT) in an inter-laboratory international collaborative study. The measurements were performed by the Agency for Health and Food Safety (AGES), Department for Molecular Biology and Microbiology (Vienna, AT) that has demonstrated competence in GMO measurements and adhering to ISO/IEC 17025:2017 [12].

DNA was extracted from 200 mg samples taken from ERM-BF443a, ERM-BF443b, ERM-BF443c, ERM-BF443d and ERM BF443e, using a validated Cetyltrimethylammonium bromide (CTAB) based DNA extraction method. Gel electrophoresis was used to check the integrity of the DNA. None of the samples showed DNA degradation (data not shown).

After the extraction, the DNA was diluted in a TE-low 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 quantitative PCR test was calibrated with genomic DNA extracted from pure GMB151 soya bean powder. For the calibration curve of the soya bean specific gene, the DNA was used undiluted (approximately 300 ng DNA per 25 mL reaction) and diluted up to 200-fold. For the calibration curve of the transgene, the DNA was used in concentration of approximately 30 ng DNA per 25 mL reaction and was then subsequently diluted up to 1000-fold. The efficiency of the amplification was assessed from the slope of the regression line between the calibrants' mass fractions of GMB151 soya bean event and 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 GMB151 soya bean event are shown in Table 3.
The quantitative PCR measurements confirmed that the mass fractions of the GMB151 soya bean event in the mixed materials ERM-BF443c, d and e were consistent with the gravimetrical approach used for their preparation. No independent calibration was carried out and therefore the data in Table 3 can only be used for confirmation of the consistency of the powder dilutions during processing. No bias was found for PCR results for ERM-BF443c, d and e with the respective certified values (Section 7).

**Table 3:** Quantification of the GMB151 soya bean mass fraction in the CRMs by event-specific quantitative PCR using genomic DNA from pure GMB151 soya bean seed powder for calibration

| CRM | GMB151 soya bean mass fraction [g/kg] | $U$ ($k$ = 2) [g/kg] |
|---|---|---|
| ERM-BF443a | < 0.04 [1) 2)] | - |
| ERM-BF443b | 1034 [1)] | 28 [6)] |
| ERM-BF443c | 1.1 [3)] | 0.1 [7)] |
| ERM-BF443d | 10.3 [4)] | 0.3 [7)] |
| ERM-BF443e | 102 [5)] | 3 [7)] |

[1)] Mean of 2 samples (extraction replicates) from each of 5 randomly selected units ($N$ = 5, $n$ = 2), with each sample measured in 3 quantitative PCR replicates.
[2)] The value was below the LOD determined during measurement (0.04 g/kg).
[3)] Mean of 3 samples (extraction replicates) from each of 12 randomly selected units ($N$ = 12, $n$ = 3), with each sample measured in 3 quantitative PCR replicates.
[4)] Mean of 2 samples (extraction replicates) from each of 12 randomly selected units ($N$ = 12, $n$ = 2), with each sample measured in 3 quantitative PCR replicates.
[5)] Mean of 2 samples (extraction replicates) from each of 15 randomly selected units ($N$ = 15, $n$ = 2), with each sample measured in 3 quantitative PCR replicates.
[6)] Expanded uncertainty with a coverage factor $k$ = 2 based on the standard deviation of measurements performed under repeatability conditions
[7)] Expanded uncertainty with a coverage factor $k$ = 2 based on the standard deviation of measurements performed under intermediate precision conditions.

# 4 Homogeneity

A key requirement for any reference material produced as a batch of units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the variation of measurement results. Consequently, ISO 17034 [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 measurements (Sections 3.5 and 5.1). As the measurement results were obtained under intermediate precision conditions on units taken randomly from the entire batch and analysed in a randomised order they were as well suited to investigate the CRM homogeneity. Homogeneity of the blank material was demonstrated by the test for the purity of the raw materials (Section 3.1). The homogeneity of ERM-BF443b is related to the purity study based on results from BBCC (Gent, BE). The batch was considered to be homogeneous (Section 3.1).

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample size is respected, but determines the minimum size of sample that is representative for the whole unit.

## 4.1 Between-unit homogeneity

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

The number of units selected corresponds to approximately the cube root of the total number of units produced. For ERM-BF443c and ERM-BF443d, 12 units were selected and 15 units for ERM-BF443e, to facilitate both the homogeneity studies and the short-term stability study. These units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. Random stratified sampling involves dividing the batch into 12 and 15 groups respectively (with a similar number of units in each group) and selecting one unit randomly from each group. For ERM-BF443c, three independent samples (extraction replicates) were taken from each selected unit whilst for the CRMs with higher GM mass fractions, ERM-BF443d and ERM-BF443e, two independent samples (extraction replicates) were taken from each selected unit, and analysed by quantitative PCR. The measurements for GM event GMB151 were performed under intermediate precision conditions (eight different days). Consequently, day-to-day effects can occur that could mask the between-unit variation. Significant differences between the day means were checked using analysis of variance (ANOVA) at a 95 % confidence level. The measurements were performed in a randomised manner to separate a potential drift in the measurement results from a potential trend in the filling sequence. The results are shown as graphs in Annex A.

No significant day-to-day effects were found for the GMB151 mass fraction in ERM-BF443c and ERM-BF443d. However, significant day-to-day effects were found for the GMB151 mass fraction in ERM-BF443e. In this case, data were first normalised to the respective day mean and tests for outlying sample means and trend in the filling sequence were performed. No outliers and trend were detected. Subsequently, between-unit homogeneity of ERM-BF443e was assessed by two-way ANOVA on the original, non-normalised data using Statistica 13 (Dell Software, Round Rock, USA). Two-way ANOVA differentiates between sample-to-sample, day-to-day, and random effects. The between-unit variation ($s_{bb}$) is separated from the within-unit variation ($s_{wb}$), which is equivalent to the method intermediate precision.

Regression analyses were performed to evaluate potential trends in the measurement sequence as well as trends in the filling sequence. No trends in the filling sequence or the measurement sequence of ERM-BF443c, ERM-BF443d and ERM-BF443e were observed at a 95 % confidence level.

These three datasets were assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. No outlying individual results and outlying unit means were detected.

Quantification of between-unit inhomogeneity for ERM-BF443c and ERM-BF443d 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 for the whole unit.

Evaluation by ANOVA requires mean values per unit, which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per unit was visually tested using histograms and normal probability plots. Too few data are available for the unit means to make a clear statement of the distribution. Therefore, it was checked visually whether all individual data follow a unimodal distribution using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. The individual values of the data sets of ERM-BF443c, ERM-BF443d and ERM-BF443c followed a unimodal distribution.

It should be noted that $s_{bb,\,rel}$ and $s_{wb,\,rel}$ are estimates of the standard deviations and are therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups ($MS_{within}$), resulting in a negative number under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be less 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.* [13]. $u^*_{bb}$ is comparable to the limit of detection (LOD) of a measurement method yielding the maximum degree of inhomogeneity that might be undetected by the given study setup.

Method intermediate precision ($s_{wb,\,rel}$) (equivalent to the within-unit standard deviation), between-unit standard deviation ($s_{bb,\,rel}$) and $u^*_{bb,\,rel}$ were calculated as:

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

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

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

| | |
|---|---|
| $MS_{within}$ | mean of squares within-unit from ANOVA |
| $MS_{between}$ | mean of squares between-unit from ANOVA |
| $\bar{y}$ | mean of all results of the homogeneity study |
| $n$ | mean number of replicate analysis 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. The resulting values from the above equations were converted into relative uncertainties.

**Table 4:** Results of the homogeneity studies

| ERM | $s_{wb,\,rel}$ [%] | $s_{bb,\,rel}$ [%] | $u^{*}_{bb,\,rel}$ [%] | $u_{bb,\,rel}$ [%] |
|---|---|---|---|---|
| ERM-BF443c | 13.6 | n.c. | 4.2 | 4.2 |
| ERM-BF443d | 7.7 | n.c. | 3.5 | 3.5 |
| ERM-BF443e | 4.0 | 1.5 | 2.4 | 2.4 |

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

The homogeneity studies 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 size

The within-unit homogeneity is correlated to the minimum sample size. The minimum sample size is the minimum amount of sample that is, for a given measurand, representative of the whole unit and thus should be used in an analysis. Using sample intakes equal to or above the minimum sample size 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 variation of measurement results at this sample intake.

ERM-BF443a and ERM-BF443b 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 quantitative PCR measurements carried out on these two powders it was concluded that also for these two pure materials the suitable minimum sample intake for quantitative PCR is 200 mg.

# 5 Stability

Time, temperature, light (including ultraviolet radiation) and water content were regarded as the most relevant influences on the stability of the materials. The influence of ultraviolet and visible light was minimised by storing the material in brown glass vials, which reduce light exposure. In addition, materials are stored in the dark and dispatched in boxes, thus removing any possibility of degradation by light. The water content was adjusted to an optimum during processing. 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 dispatch of the materials to the customers (short-term stability). During transport, especially in summer, temperatures 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-BF443e material was selected for the short-term stability study because it is a mixture of both GM and non-GM base materials and allows assessment of the stability of each base material. Moreover, the mixture with the highest GM mass fraction provides the best method intermediate precision ($s_{wb,rel}$). The short-term stability study was carried out using an isochronous design [14]. In this approach, units of ERM-BF443e were stored for a certain time at different temperature conditions. Afterwards, the units were moved to conditions where further degradation can be assumed negligible (reference conditions). At the end of the isochronous storage, samples taken from the units are analysed simultaneously under intermediate precision conditions. Analysis of the material (after various exposure times and temperatures) under intermediate precision conditions greatly improves the sensitivity of the stability tests.

## 5.1 Short-term stability study

In the short-term stability study the conditions for dispatch of the material to the customers were established. To this end, units were stored at 4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Five units per storage time were selected using a random stratified sampling scheme. From each unit, two samples were measured by quantitative PCR. The measurements were performed under intermediate precision conditions (eight different days, measurements performed together with homogeneity studies), and a randomised sequence was used to differentiate any potential drift in the measurement results from a potential trend over storage time.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test on a confidence level of 99 %. No statistical outliers were detected for GMB151 soya bean and all data were retained for the estimation of $u_{sts}$.

In addition, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated, to test for potential increases or decreases of GMB151 soya bean mass fraction due to 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 results of the measurements are shown in Annex B.

No significant degradation during dispatch even at 60 °C was observed. Therefore, the material can be transported at ambient conditions without special precautions.

## 5.2 Long-term stability study

Long-term storage conditions and shelf life guaranteeing the stability of the material and the certified values were established.

Data from the post-certification stability monitoring programme for GMO CRMs were available. Previously released soya bean powder CRMs were analysed for their GM mass fraction on 44 occasions over a period of eleven years. At each time point measurements were performed on units stored at 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 ratio of samples from 4 °C and -70 °C.

To verify that the data obtained from stability monitoring of similar GMO CRMs produced and stored in the same way could be used to estimate the stability uncertainty contribution for ERM-BF443, the data of the short-term stability study were compared to the stability monitoring data. The data of the 4 °C short-term stability study 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 evaluated. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %.

In addition, the data were plotted against storage time and linear regression lines of GM mass fraction versus time were calculated. The slope of the regression line was tested for statistical significance (loss/increase due to storage).

The results of the long-term stability measurements are shown in Annex C.

No statistical outliers were detected for GMB151 soya bean, all data were retained for the estimation of $u_{lts}$. No statistically significant trend was detected on a 95 % confidence level for the tested temperature. The material can therefore be stored at 4 °C.

## 5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out 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 within an uncertainty to be estimated.

The uncertainties of stability during dispatch and storage were estimated, as described in [14]. 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:

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

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

$s_{rel}$ 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 °C)
$t_{sl}$ chosen shelf life (24 months at 4 °C)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of stability during dispatch. This was estimated from the 60 °C study. The uncertainty describes the possible change during a dispatch at 60 °C lasting for one week.
- $u_{lts,rel}$, the uncertainty of stability during storage. This uncertainty contribution was estimated from the stability monitoring programme for soya bean GMO CRMs. The uncertainty contribution describes the possible degradation during storage for 24 months 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

| CRM | $u_{sts,rel}$ [%] | $u_{lts,rel}$ [%] |
|---|---|---|
| ERM-BF443 | 0.7 | 0.6 |

After the certification study, the materials will be included in the JRC's regular stability monitoring programme, to control its further stability.

# 6 Characterisation

The material characterisation is the process of determining the property value of a reference material.

This was based on a primary method of measurement (weighing) confirmed by independent analysis (using PCR). Gravimetric mixing was chosen as the method of choice. The three CRMs under the label code ERM-BF443c, ERM-BF443d and ERM-BF443e are soya bean powder materials processed from non-GM and GM seeds. While ERM-BF443a is prepared from the pure blank material and ERM-BF443b from the pure GM material, the other CRMs of the ERM-BF443 series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF443 is certified for the mass fraction of GMB151 soya bean event.

## 6.1 Purity of the base materials

The purity of the GM and non-GM batches used for the processing of these powders was investigated to calculate the certified value.

The purity of the GM GMB151 soya bean material was based on information from BBCC (Gent, BE) (Section 3.1).

The powder used for the production of ERM-BF443a did not contain traces of GMB151 soya bean above the LOD of the real-time PCR method used (Section 3.1 and 3.5). The certified value for ERM-BF443a is therefore with 95 % confidence interval below the LOD of the real-time PCR method used, as determined during homogeneity, short-term stability and confirmation measurements.

The eventual adventitious presence of other GM events in both the GM and non-GM soya bean powders was verified by using a qualitative PCR-based ready-to-use multi-target analytical system for GM detection developed by JRC (Ispra, IT) [15]. This test was performed at the JRC by using a pre-spotted 96-well plate containing primers and probes for simultaneous detection of 15 individual specific GM soya bean events (DP356043, A2704, FG72, A5547, GTS40-3-2, CV127, MON 87701, DAS44406, MON87705, DAS68416, MON87708, DAS81419, MON87769, DP305423 and MON89788) and the primers and probes for the taxon-specific assay for soya bean (Lec). Any stacked events derived from the single-insert GMOs included in the system would also be detected. The results indicated that the non-GM soya bean powder used for the production of ERM-BF443 did not contain any of the above tested GM events and was only positive for the taxon-specific detection for soya bean (Lec). For the GM soya bean powder the results showed positive for the taxon-specific detection for soya bean (Lec) and the soya bean GM events GTS40-3-2, MON87701 and MON89788 although at a very low concentration, estimated to be < LOD of the related PCR methods.

Since no evidence of quantifiable contamination was found in both base materials, 100 % purity was used for the calculation of the certified mass fraction of GMB151 soya bean in the powder mixtures. The half-width of the difference between the measured purity of > 99 % (Section 3.1) and the 100 % purity was taken into account in the uncertainty calculation.

## 6.2 Mass fractions and their uncertainties

The certified mass 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 purity with regard to the GMB151 soya bean event. The values were calculated according to the following equations:

$$w_{GM} = \frac{m_{GM,\,dry}}{m_{GM,\,dry} + m_{nonGM,\,dry}} \cdot 1000$$ **Equation 6**

$$m_{GM,\,dry} = m_{GM,\,wet} \cdot \left(1 - w_{water,\,GM}\right)$$ **Equation 7**

$$m_{nonGM,\,dry} = m_{nonGM,\,wet} \cdot \left(1 - w_{water,\,nonGM}\right)$$ **Equation 8**

| | |
|---|---|
| $w_{GM}$ | GM mass fraction of the powder [g/kg] |
| $m_{GM,\,dry}$ | mass of the GM powder corrected for its water mass fraction [g] |
| $m_{nonGM,\,dry}$ | mass of the non-GM powder corrected for its water mass fraction [g] |
| $m_{GM,\,wet}$ | mass of the GM powder used for the dilution (not corrected for its water mass fraction) [g] |
| $m_{nonGM,\,wet}$ | mass of the non-GM powder used for the dilution (not corrected for its water mass fraction) [g] |
| $w_{water,\,GM}$ | water mass fraction of the GM powder [g/g] |
| $w_{water,\,nonGM}$ | water mass fraction of the non-GM powder [g/g] |

In Table 6, the data supporting the calculation of the mass fractions of the GMB151 soya bean event are summarised.

**Table 6:** Subsequent mixing of GM GMB151 soya bean seed powder (ERM-BF443b, ERM-BF443e, or ERM-BF443d) with non-GM powder (ERM-BF443a) to prepare ERM-BF443e, ERM-BF443d and ERM-BF443c materials.

| ERM-BF443 produced | GM powder used[1)] | | | Non-GM powder [1)] | | Mixtures |
|---|---|---|---|---|---|---|
| | Mass fraction [g/kg] | Water mass fraction ± $U$ ($k$ = 2) [g/kg] | Mass [g] | Water mass fraction [5)] ± $U$ ($k$ = 2) [g/kg] | Mass [g] | Calculated GM mass fraction [g/kg] |
| ERM-BF443e | 1000.0 [2)] | 23.0 ± 1.5 | 398.71 | 26.1 ± 1.7 | 3601.12 | 100 |
| ERM-BF443d | 100 [3)] | 27.9 ± 1.8 [3)] | 400.54 | 26.1 ± 1.7 | 3599.31 | 10.0 |
| ERM-BF443c | 10.0 [4)] | 27.5 ± 1.8 [4)] | 400.49 | 26.1 ± 1.7 | 3599.46 | 1.00 |

[1)] Calculations of the certified mass fraction of GMB151 soya bean in the powder mixtures are based on a 100 % purity of the non-GM and GM base materials

[2)] Pure GM powder ERM-BF443b was used for the preparation of ERM-BF443e

[3)] GM powder mixture ERM-BF443e was used for the preparation of ERM-BF433d, water mass fraction of ERM-BF443e determined by (V-KFT)

[4)] GM powder mixture ERM-BF443d was used for the preparation of ERM-BF433c, water mass fraction of ERM-BF443d determined by (V-KFT)

The uncertainties of the certified mass fractions ($u_{char}$) of GMB151 soya bean have several components i.e. the uncertainty of the mass determination ($u_{char,1}$), the uncertainty of the water mass fraction analysis ($u_{char,2}$), and the uncertainties of the purity determination of the non-GM and GM base powders ($u_{char,3}$ and $u_{char,4}$). Based on measurement data from BBCC (Gent, BE) the purity was higher than 99 % (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 GMB151 soya bean in ERM-BF443

| ERM-BF443 | 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-BF443a | 0 | n.a. [5)] | n.a. [5)] | 0.0115 | n.a. [5)] | 0.0115 |
| ERM-BF443b | 1000 | n.a. [5)] | n.a. [5)] | n.a. [5)] | 2.8819 | 2.8819 |
| ERM-BF443c | 1 | 0.0011 | 0.0018 | 0.0115 | 0.0029 | 0.0121 |
| ERM-BF443d | 10 | 0.0093 | 0.0157 | 0.0115 | 0.0288 | 0.0360 |
| ERM-BF443e | 100 | 0.0657 | 0.1282 | 0.0115 | 0.2881 | 0.3223 |

[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 purity estimation of the non-GM base material (LOD = 0.04 g/kg), based on the half-width of the interval between 0 and 0.04 g/kg, divided by the square root of 3 (rectangular distribution)
[4)] Standard uncertainty of the purity estimation of the GM base material (> 99 %), based on the half width of the interval between 99 % 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 3.5). Gel-electrophoresis proved that the DNA was not degraded during processing of the CRM.

# 7 Value Assignment

Certified values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Full uncertainty budgets in accordance with ISO 17034 [1] and ISO Guide 35 [2] were established.

The certified values are based on the masses of dried powder of GM seeds and non-genetically modified seeds used in the gravimetrical preparation. The masses of the powders were corrected for their respective water mass fractions during the preparation of the materials (Table 6).

The assigned uncertainty consists of uncertainties relating to characterisation ($u_{char}$), potential between-unit inhomogeneity ($u_{bb}$), and potential degradation during transport ($u_{sts}$), and long-term storage ($u_{lts}$). 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:

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

- $u_{char}$ 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 blank material, the LOD (0.04 g/kg) of the PCR method was used to describe the 95 % confidence interval of the certified mass fraction of GMB151 soya bean. This was supported by the high purity of the (non-GM) material and the absence of any mixing step; calculating the $U_{CRM}$ for the blank material on the basis of the only quantifiable standard uncertainty ($u_{char,3}$) gives a value of $U$ = 0.03 g/kg (assuming $k$ = 2), which is below the LOD. The LOD is, therefore, a conservative estimate of the uncertainty of the certified value. With 95 % confidence, the true GMB151 soya bean mass fraction of the material is below 0.04 g/kg. Based on the high purity, the certified value for this material is artificially set at 0 g/kg. The asymmetric expanded uncertainty is based on the difference between the certified value (0 g/kg) and the LOD (0.04 g/kg).

For the pure GM material, the purity of the GM seed batch (> 990 g/kg) (Section 3.1) was used to describe the 95 % confidence interval of the certified mass fraction of the event GMB151 soya bean. Calculating $U_{CRM}$ for the pure GM material on the basis of the only quantifiable standard uncertainty ($u_{char,4}$) gives a value of $U$ = 6 g/kg (assuming $k$ = 2), which is less than the difference between the certified value (1000 g/kg) and the purity (> 990 g/kg). The statistically calculated purity is, therefore, a conservative estimate of the uncertainty of the certified value. The certified value is artificially set at 1000 g/kg and the related asymmetric expanded uncertainty is based on the difference between certified value (1000 g/kg) and the purity (> 990 g/kg).

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-BF443c, d and e.

A $k$-factor of 2 was applied to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 8.

**Table 8:** Certified values and their uncertainties for ERM-BF443

| GMB151 soya bean / ERM-BF443 | 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-BF443a | 0 [1)] | 0.0115 | n.a. [4)] | n.a. [4)] | n.a. [4)] | - 0 [5)] +0.04 |
| ERM-BF443b | 1000 [2)] | 2.8819 | n.a. [4)] | n.a. [4)] | n.a. [4)] | + 0 [6)] - 10 |
| ERM-BF443c | 1.00 | 0.0121 | 0.0420 | 0.0070 | 0.0060 | 0.09 |
| ERM-BF443d | 10.0 | 0.0360 | 0.3498 | 0.0700 | 0.0600 | 0.8 |
| ERM-BF443e | 100 | 0.3223 | 2.3992 | 0.6998 | 0.5998 | 6 |

[1)] The certified value is based on the LOD (0.04 g/kg) of the event-specific PCR method. No contamination was detected in this material when using an event-specific qPCR assay targeting the GMB151 soya bean event. With 95 % confidence, the true GMB151 soya bean mass fraction of the material is below 0.04 g/kg.
[2)] The certified value is based on the genetic purity of the soya bean seed powder with regard to GMB151. With 95 % confidence, the true GMB151 mass fraction of the material is above 990 g/kg.
[3)] Expanded ($k$ = 2) and rounded uncertainty; uncertainties are always rounded up [16] and in a way that the rounding error corresponds to 3 % to 30 % of the uncertainty
[4)] n.a.: not applicable
[5)] The asymmetric uncertainty is based on the 95 % confidence interval of the LOD. With 95 % confidence, the true GMB151 mass fraction of the material is therefore between 0 and 0.04 g/kg. If using ERM BF443a for calibration the value 0 g/kg with an expanded uncertainty of + 0.04 / - 0 g/kg should be used. The corresponding standard uncertainty is equal to 0.04 g/kg / √3 = 0.03 g/kg.
[6)] The asymmetric uncertainty is based on the 95 % confidence interval of the purity data from BBCC (Gent, BE). With 95 % confidence, the true GMB151 mass fraction of the material is therefore between 990 and 1000 g/kg. If using ERM-BF443b for calibration the value 1000 g/kg with an expanded uncertainty of + 0 / - 10 g/kg should be used. The corresponding standard uncertainty is equal to 10 g/kg / √3 = 6 g/kg.

As no proof could be delivered regarding how the certified GM powder mass fractions are related to the corresponding transgenic and species-specific DNA copy number ratios, the user is reminded that JRC only certifies these materials for their mass fraction of event. Additionally, one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements on unknown samples as DNA- and/or protein-based quantification of GMOs may vary with the particular matrix and the species variety tested.

# 8 Metrological traceability and commutability

## 8.1 Metrological traceability

**Identity**

The identity of the measurand is based on the documentary traceability to the GMB151 soya bean event (Biosafety Clearing House, record ID 115763) [7].

**Quantity value**

The traceability chain for the certified values for the pure non-GM and GM CRMs, ERM-BF443a and ERM-BF443b respectively, are based on the genetic purity assessment using a validated event-specific GMB151 soya bean quantitative PCR method and verified equipment.

The traceability chain for the certified values for the mixtures in ERM-BF443c, d and e is based on the SI-traceable calibration of the balances used and a thorough control of the weighing procedure. The certified values are therefore traceable to the International System of Units (SI).

## 8.2 Commutability

The concept of commutability of a reference material is defined by the VIM [16] as:

*"property of a reference material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in this material, obtained according to two given measurement procedures, and the relation obtained among the measurement results for other specified materials"*

The certified value of this CRM is structurally defined via its DNA sequence. The CRM was prepared gravimetrically from pure non-GM and GM seed powder with the aim to implement the mass fraction based thresholds set in the corresponding EU legislation for food and feed.

The CRMs are intended for quality control or calibration of real-time PCR measurements of the soya bean GM event GMB151 in food and feed. Consequently, in combination with the measurement method validated by the EURL-GMFF [17], this set of CRMs is establishing the arbitrary reference system required for quantification of GMB151 soya bean.

Commutability of this reference material does not need to be assessed.

# 9 Instructions for use

## 9.1 Safety information

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 any unit immediately after taking a sample.

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

## 9.3 Minimum sample size

The minimum sample size for DNA extraction is 200 mg.

ERM-BF443a and ERM-BF443b are pure non-GM and GM materials, respectively. Therefore, the minimum sample size 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 intended use of these materials is for calibration or quality control of GMB151 soya bean detection methods. As with any reference material, they can be used for establishing control charts or validation studies.

The user is reminded that this reference material is certified for its GMB151 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. It is recommended that different concentration levels of ERM-BF443 be used for calibration and quality control.

Comparing a measurement 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 [18]).

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

- Calculate the absolute difference between mean measured value and the certified value ($\Delta_{meas}$).
- Combine the measurement uncertainty ($u_{meas}$) with the uncertainty of the certified value ($u_{CRM}$): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty ($U_{\Delta}$) from the combined uncertainty ($u_{\Delta}$) 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.

# 10 Acknowledgements

The authors would like to acknowledge the support received from colleagues of JRC for the processing, organising of stability studies, measuring, reviewing of the certification project and distribution of these CRMs.

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# Annexes

**Annex A: Results of the homogeneity measurements**

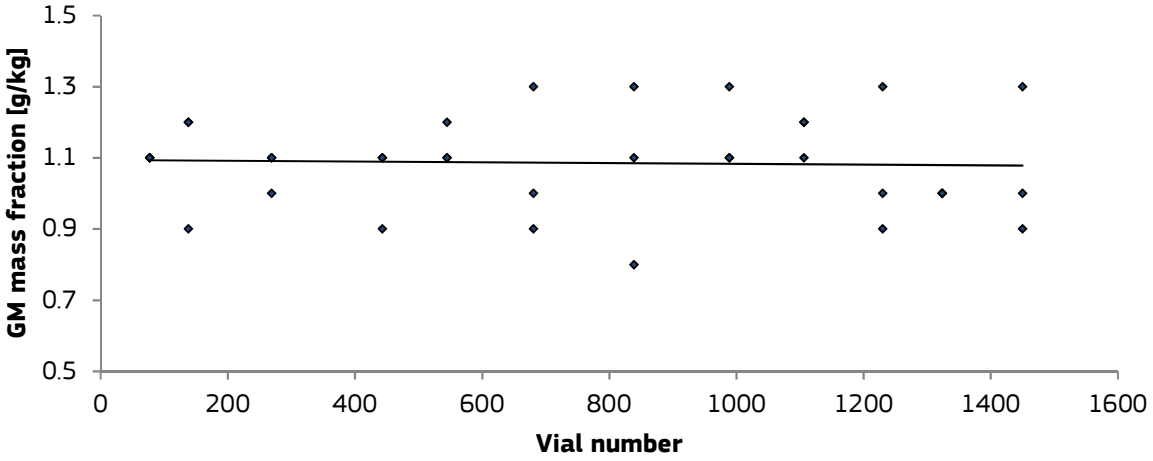


**Figure A1**: Quantitative PCR measurement results for ERM-BF443c. Three samples (extraction replicates) were measured from each of 12 randomly selected units ($N$ = 12, $n$ = 3), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

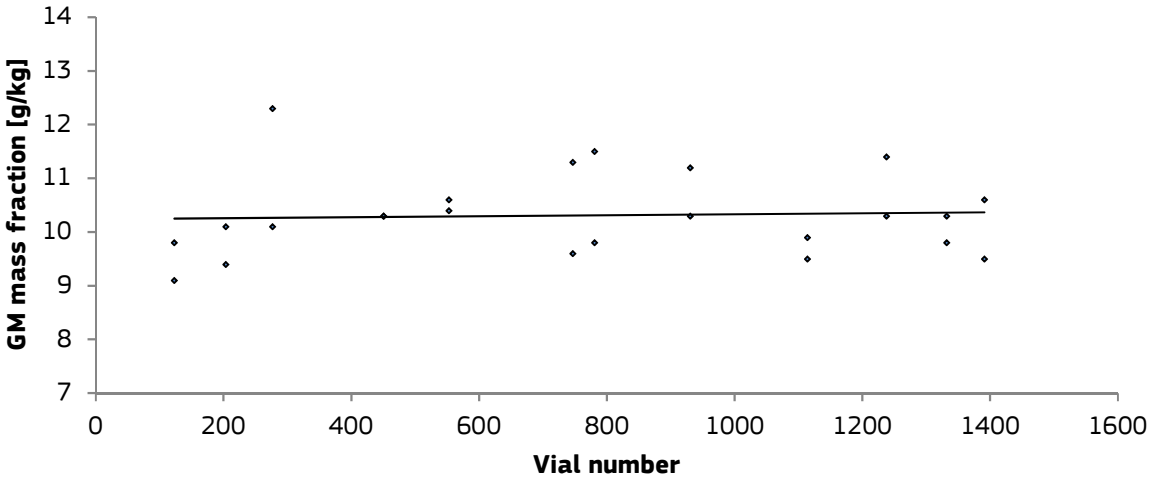


**Figure A2**: Quantitative PCR measurement results for ERM-BF443d. Two samples (extraction replicates) were measured from each of 12 randomly selected units ($N$ = 12, $n$ = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

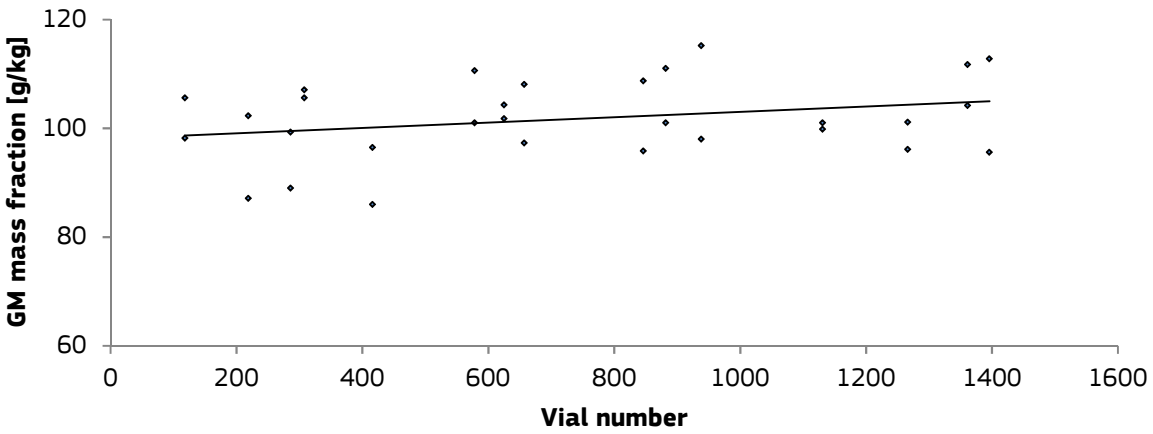


**Figure A3**: Quantitative PCR measurement results for ERM-BF443e. Two samples (extraction replicates) were measured from each of 15 randomly selected units ($N$ = 15, $n$ = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

**Annex B: Results of the short-term stability measurements**

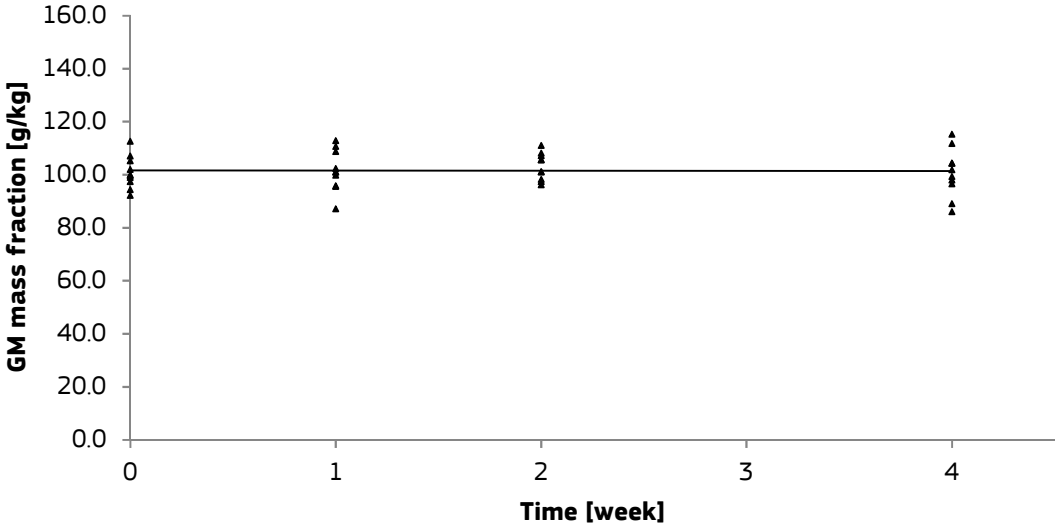


**Figure B1**: Quantitative PCR measurement results for ERM-BF443e during short-term stability testing at 4 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected units ($N$ = 5, $n$ = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

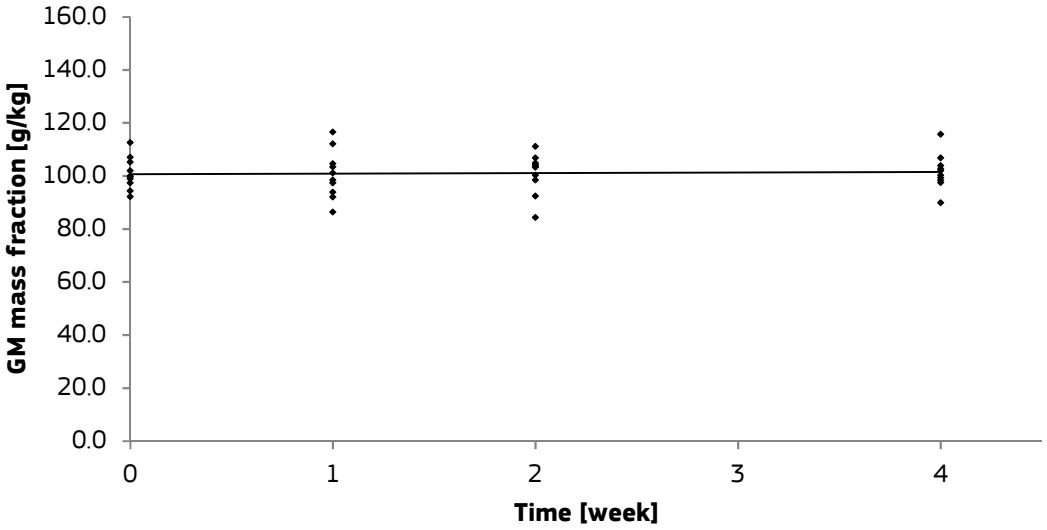


**Figure B2**: Quantitative PCR measurement results for ERM-BF443e during short-term stability testing at 18 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected units ($N$ = 5, $n$ = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

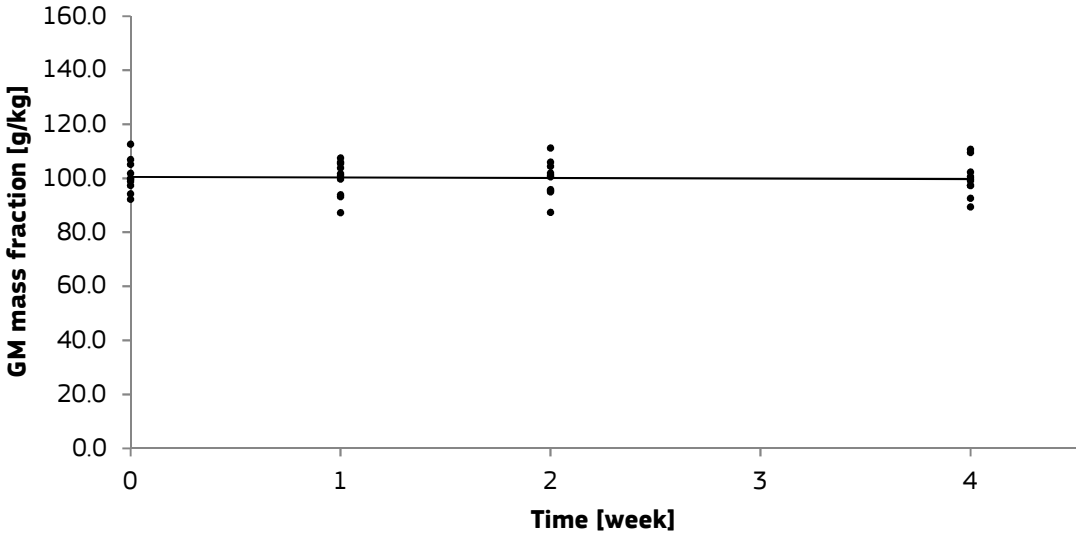


**Figure B3**: Quantitative PCR measurement results for ERM-BF443e during short-term stability testing at 60 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected units ($N$ = 5, $n$ = 2), with each sample measured in 3 quantitative PCR replicates under intermediate precision conditions. The straight line is a least-squares linear regression for all data points.

**Annex C: Results of the long-term stability measurements**

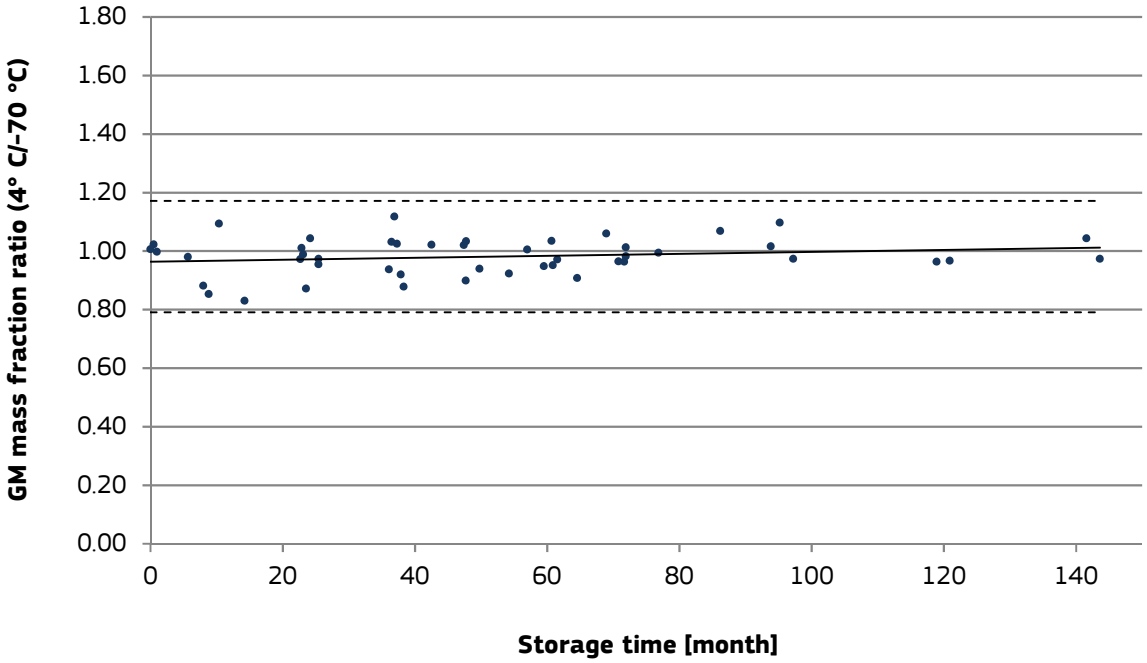


**Figure C1**: Quantitative PCR measurement results of ERM-BF443e (1, 2 and 4 weeks) and ERM-BF410, ERM-BF425, ERM-BF426, ERM-BF432, ERM-BF436 and ERM-BF437 (data from the post-certification monitoring). The dashed lines give the limits of 3*s* obtained for the measurement results. The straight line is a least-squares linear regression for all data points.

European Commission

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