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European Reference Materials

CERTIFICATION REPORT

The certification of different mass fractions of AV43-6-G7 in potato tuber powder:

Certified Reference Materials ERM®-BF431a, ERM®-BF431b, ERM®-BF431c, ERM®-BF431d and ERM®-BF431e

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The certification of different mass fractions of AV43-6-G7 in potato tuber powder:

Certified Reference Materials ERM®-BF431a, ERM®-BF431b, ERM®-BF431c, ERM®-BF431d and ERM®-BF431e

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Summary

This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF431a, b, c, d and e, matrix materials certified for their AV43-6-G7 mass fractions. The material has been produced following ISO Guide 34:2009 [1].

Genetically modified (GM) tubers of the potato event AV43-6-G7 and of a non-GM potato variety were dried and ground to obtain GM and non-GM potato powders. Beside these two pure materials gravimetric mixtures of non-GM and GM potato powder were prepared by dry-mixing.

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

The certified value was obtained from the gravimetric preparations, taking into account the purity of the base materials and their water mass fraction. The certified values were confirmed by event-specific real-time PCR as independent confirmation method (measurements within the scope of accreditation to ISO/IEC 17025:2005 [3]).

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

The materials are intended for the calibration or quality control of AV43-6-G7 potato identification and quantification methods. As any reference material, they can also be used for control charts or validation studies. The CRMs are available in glass vials containing at least 1 g of dried potato tuber powder and closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.

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

The following values were assigned:

	AV43-6-G7 Mass Fraction [1]	
	Certified value [2] [g/kg]	Uncertainty [3] [g/kg]
ERM-BF431a	0	-
ERM-BF431b	1000	-
ERM-BF431c	9.9	1.3
ERM-BF431d	40	5
ERM-BF431e	99	10

1) Genetically modified potato with the unique identifier AV43-6-G7.

2) Mass fraction of AV43-6-G7 potato based on the masses of genetically modified AV43-6-G7 potato tuber powder and non-modified potato tuber powder and their respective water content. The certified values and their uncertainties are traceable to the International System of Units (SI).

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

Disclaimer

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

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Glossary

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

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

1 Introduction

1.1 Background: need for the CRM

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

Avebe Agro (Foxhol, NL) has developed the genetically modified (GM) potato event AV43-6-G7 which received the unique identifier code AVE-436G7-1 following Commission Regulation (EC) No 65/2004 [6]. In 2010, Avebe Agro asked the Institute for Reference Materials and Measurements (IRMM, Geel, BE) to produce a reference material for the quantification of AV43-6-G7 potato. AV43-6-G7 is a starch potato variety intended to be used by the starch industry and contains one insert of an inverted repeat construct of the granule bound starch synthase (GBSS) gene in order to down-regulate the synthase [7]. According to the information provided by Avebe Agro, the genetically modified AV43-6-G7 potato contains a single copy of a RNAi-construct of GBSS and four intact copies of the GBSS are still present. AV43-6-G7 potato is intended to be used for processing into starch. The Certified Reference Material (CRM) produced by IRMM has been named ERM-BF431 and is composed of a set of five CRMs with different mass fractions of AV43-6-G7 potato and relates to application EFSA-GMO-NL-2009-69 according to Commission Regulation (EC) No 1829/2003 by the applicant BASF Plant Science Company GmbH, Ludwigshafen, DE.

1.2 Choice of the material

The set of CRMs ERM-BF431 was produced from dried and ground GM tubers and non-GM tubers. Tubers were selected as raw material as they were considered to be closest to the food and feed samples checked for labelling requirements in Europe [5].

1.3 Design of the project

Beside a non-GM and GM pure material, gravimetric mixtures of non-GM and GM potato powder were prepared by dry-mixing, a first material by mixing non-GM and GM potato powder and a second and third one by further dilution of the mixture with non-GM potato powder.

The different mass fractions of ERM-BF431 were certified using a gravimetric approach and details are described in this certification report.

2 Participants

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM and to ISO/IEC 17025 for the quantification of GMOs, BELAC No. 268-TEST)

3 Material processing and process control

3.1 Origin of the starting material

For the preparation of the CRMs, Avebe Agro supplied non-GM potato tubers of the variety Karnico (*Solanum tuberosum L. cv*. Karnico) and GM potato tubers of the event AV43-6-G7 to IRMM. After arrival, the potato tubers were stored for less than 24 hours at (4 ± 3) °C in the dark until used for processing.

The purity of the delivered non-GM and GM potato tuber batches had been controlled by Avebe Agro. During the growing season, leave samples from the potato plants were checked and DNA extracted from the non-GM variety Karnico tested negative in PCR, while the plants of AV43-6-G7 tested positive in PCR. The tubers were harvested separately and about 10 % of them were analysed for the presence of amylose in the tubers with Lugol's staining solution [8]. Surface chips from non-GM potato tubers turned dark blue upon reaction with the iodine solution as they contain about 25 % amylose, while those from the GM tubers, which largely lack amylose, turned red/brown.

Before processing, the purity of the batches was tested additionally at IRMM. Each potato tuber was tested individually by iodine staining [8]. 737 non-GM potato tubers turned blue to indicate the presence of amylose and were used for the processing of the non-GM powder. 223 GM potato tubers turned red/brown and indicated to largely lack amylose. These tubers were used for the processing of the GM powder. Prior to the use, the staining method [8] had been in-house validated by IRMM for detection of starch modified potatoes. This validation had been carried out on AM04-1020-4 modified potatoes and their non-modified counterpart [9]. The testing results confirmed that the 100 % purity of the non-GMO and GMO potato batches.

3.2 Processing

The GM and non-GM base materials were processed separately. Due to the total mass of the non-GMO batch, processing was carried out in two individual batches which were merged later on. Cross-contamination and contamination with foreign DNA were avoided applying systematic cleaning, clean laboratory clothing and further measures to prevent cross-contamination by air. All contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to exposure to the base materials. An in-house validation study had proven beforehand that the solution degraded DNA effectively under the given conditions. If required, the base powders were stored for short time periods in closed plastic containers.

All potato tubers received by IRMM were brushed carefully and rinsed twice with tap water. Sprouts were removed. The tubers were dried with cotton and starch-free tissues and finally air dried. The tested potatoes were cut with a hand chipping machine CF-5 (Sammic, Azkoitia, ES) into rectangular strips of about 1 cm thickness and subsequently manually cut into cubes of about 1 cm^3. The cubes were then further dried for about 80 hours in a freeze drier (Epsilon 2-65D, Osterode, DE). The mass of the tubers was diminished by this procedure with about 75 %. Until further processing the dried potato cubes were packed in portions of 2 kg in plastic bags and stored at -20 °C in a container under nitrogen atmosphere.

The potato cubes were milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). Prior to this milling step the potato cubes were frozen overnight in approximately 4 kg portions in stainless steel containers immersed in liquid nitrogen. The mill was also cooled to process the cubes at a temperature below -90 °C. The slowest feeding speed of the mill was used to ensure most efficient milling with respect to the particle size

obtained. After milling, the powder was kept at (4 ± 3) °C. The GM and non-GM powders were then sieved separately with a 500 µm stainless steel mesh on a sieving machine (Russel Finex, London, UK). In case of the GM powder a coarse fraction of 0.8 g did not pass the 500 µm mesh and was discarded. For the non-GM powder a coarse fraction of 3.6 g did not pass the 500 µm mesh and was discarded. The remaining powder of each base material, which passed the sieve, was mixed in a DynaMIX CM200 (WAB, Basel, CH) for 30 min to improve equal distribution of the different types of potato tissues because the milling and sieving processes applied foster the separation of the different tissues from each other.

For the non-GM and GM powders a residual water mass fraction of respectively (25.8 ± 6.6) g/kg and (17.9 ± 4.6) g/kg with U, k = 2 was measured by volumetric Karl Fischer titration (V-KFT, Metrohm, Herisau, CH) (N = 1, n = 3) (Table 4). The particle volumes for both powders were measured based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and compared (Figure 1). The mean particle diameters (N = 1, n = 5), calculated by the PSA software, were 43 µm (s = 2 µm) for the GM powder and 40 µm (s = 4 µm) for the non-GM powder. It is important to understand that the cumulative volume distribution of particles derived from laser light scattering data is based on their equivalent spherical diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. Since most particles are presumably not perfectly spherical, the calculated volume of the particles based on their diameter is, therefore, overestimating the mean particle size. It has been concluded that the particle volume fractions of the non-GM and GM base powders were sufficiently similar to allow the processing of mixtures without introducing a bias based on the DNA extractability.

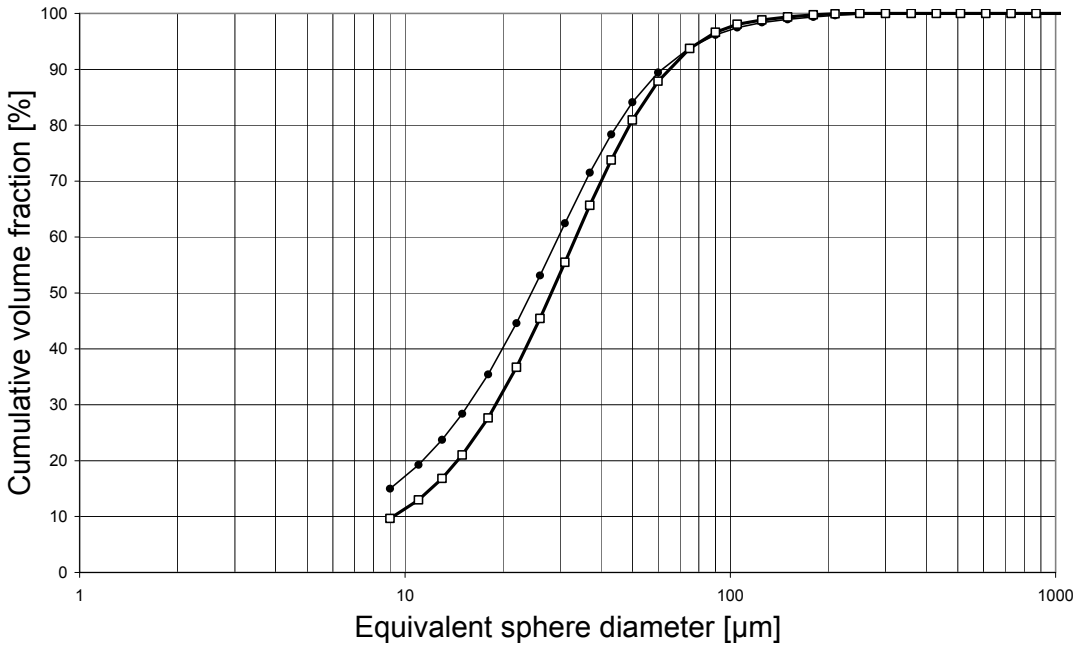

Figure 1: Accumulation of particle volume fractions in the GM powder (•) and non-GM powder (□) analysed by PSA. The mean of five replicates (N = 1, n = 5) measured is given. The total volume is set as 100 %.

The ground base materials were used to produce a blank material for AV43-6-G7 (non-GM tuber powder), a pure GM AV43-6-G7 material and three mixtures containing different mass fractions of AV43-6-G7 potato tuber powder in non-GM potato tuber powder at nominal mass fraction levels of 10, 40 and 100 g/kg. The term 'nominal' is used to discriminate between the

value targeted in the processing and the certified value assigned after completion of the certification process.

All these materials, including the blank powder, were treated according to the same procedure. The powder materials were weighed using a calibrated balance with an intermediate precision, expressed as relative standard uncertainty, of 0.1 %. Calibration of the balance is carried out on an annual basis by an external company accredited for ISO/IEC 17025 calibration services; additionally the performance of the balance is verified before use. Portions of the powder materials were weighed into containers, transferred into a propeller mixer and mixed for 2 min. During this time the powder was flushed with argon to prevent water uptake. The pure materials were processed first, followed by the mixtures. For the preparation of the mixtures the masses of the non-GM and GM powder were corrected for their respective water mass fractions. The masses which are theoretically needed to reach a certain nominal mass fraction were calculated. During certification the practically used dry masses were used to establish the certified mass fraction (Section 7.1). The material having a nominal mass fraction of 100 g AV43-6-G7/kg was produced by mixing pure GM with pure non-GM ground base materials. The materials having a nominal mass fraction of 40 and 10 g AV43-6-G7/kg were 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 mixed materials was taken into account. The gravimetric preparation formed the basis for the calculation of the mass fraction of the powders (Table 4, Section 6).

After finalisation of the mixing steps the powders were filled in 10 mL brown glass vials using an automatic filling device. The first 30 bottles of each batch were discarded as an additional precaution against carry-over contamination. Lyophilisation inserts were automatically placed in the bottle necks. Before final closure of the vials, air was evacuated in a freeze-drier and replaced by argon. The vials were finally closed inside the freeze-drier with the help of a hydraulic device and then sealed with aluminium caps to prevent accidental opening during storage and transport. Colour-coded caps were used for easy identification of the different mass fraction levels of AV43-6-G7: nominal 0 g/kg = silver (BF431a), nominal 1000 g/kg = black (BF431b), nominal 10 g/kg = red (BF431c), nominal 40 g/kg = pink (BF431d), nominal 100 g/kg = brown (BF431e), consistent with the cap colours of previous IRMM CRMs for GMOs. Each of the vials was identified by a numbered label indicating the ERM code and the unit number. Following the inventory and the selection of vials for future analysis according to a random stratified sampling scheme, the bottles were brought to a storage room for long-term storage in the dark at (4 $\pm$ 3) °C.

3.3 Process control

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

Table 1: Water mass fraction in candidate CRMs ERM-BF431 determined by V-KFT (*N* = 10, *n* = 2) with the standard deviation of the ten means of the duplicates given

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	*s*
ERM-BF431a	20.5	1.2
ERM-BF431b	15.0	1.8
ERM-BF431c	20.0	2.0
ERM-BF431d	20.7	1.3
ERM-BF431e	18.1	2.6

Five randomly selected bottles from each of the powder materials were analysed twice for their particle volume distribution (*N* = 5, *n* = 2) based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). The powders have a particle diameter below 360 µm (Figure 2). The mean particle diameters (*N* = 5, *n* = 2), calculated by the PSA software, were 32 µm (*s* = 3 µm), 34 µm (*s* = 3 µm), 34 µm (*s* = 6 µm), 34 µm (*s* = 3 µm), and 37 µm (*s* = 2 µm) for ERM-BF431a, b, c, d and e, respectively. The lower mean particle diameter measured in comparison to the non-GMO and GMO ground base material (Section 3.2) is likely to be caused by the impact of the dry-mixing.

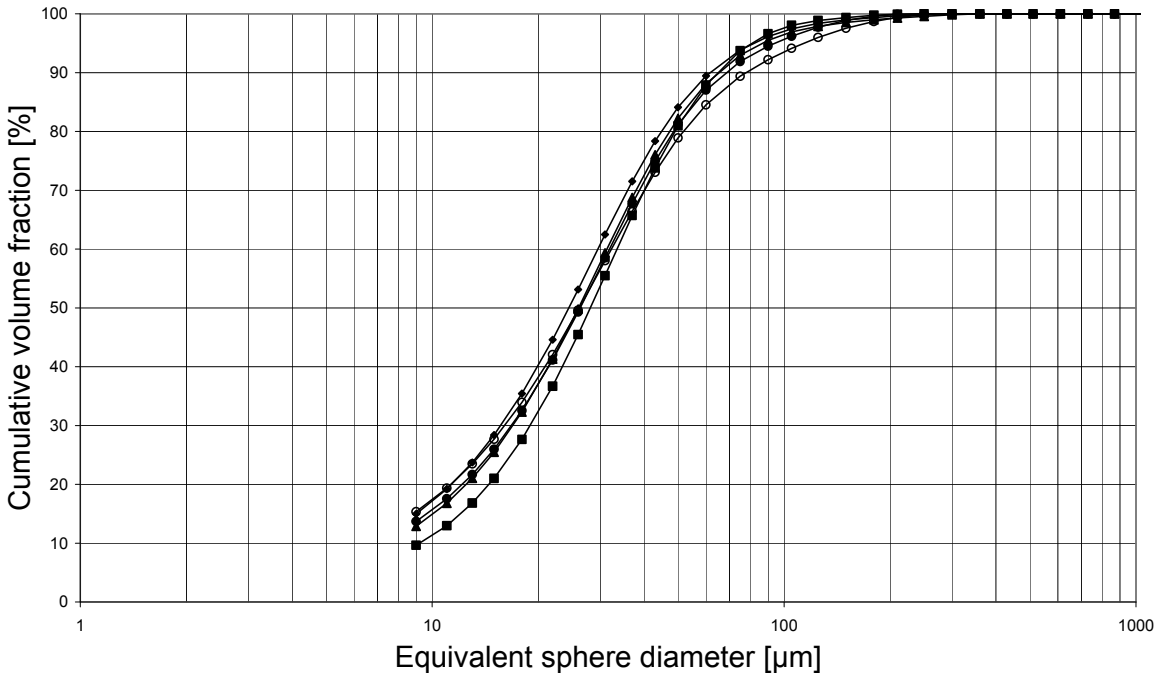

Figure 2: Accumulation of particle volume fractions in ERM-BF431a (◆), ERM-BF431b (■), ERM-BF431c (▲), ERM-BF431d (●), and ERM-BF431e (○) analysed by PSA. The mean of ten measurements (*N* = 5, *n* = 2) is given. The total volume is set as 100 %.

3.4 DNA content of the base materials

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

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

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

The ratio of the DNA mass extractable from 100 mg of GM and non-GM potato powder was found to be (1.00 ± 0.05), (N = 9 with an expanded uncertainty, k = 2). A t-test demonstrated that no significant difference between the DNA mass extracted from the GM and non-GM potato powders by the modified Ogur and Rosen method exists at 95 % confidence level.

The DNA integrity was checked by gel electrophoresis. From 200 mg samples of the processed powder materials ERM-BF431a, b, c, d, and e, DNA was extracted using a CTAB MicroSpin (S-400 HR, GE Healthcare Europe GmbH, Diegem, BE) DNA extraction method. Approximately 0.2 µg DNA were loaded on an agarose gel (mass concentration of 7.5 agarose g/L). Staining of the DNA was done with an ethidium bromide solution (500 µg/L TRIS borate EDTA buffer (T7527, Sigma-Aldrich, St. Louis, MO, US diluted to 89 mmol/L TRIS and 0.2 mmol/L EDTA, pH 8.3)). None of the samples showed DNA degradation (data not shown).

3.5 Confirmation measurements

As a control for the gravimetric preparations, the mass fraction of AV43-6-G7 potato in all four CRMs was confirmed by the confidential real-time PCR method provided by Avebe Agro targeting the transgenic DNA insertion in this potato. This method had been in-house validated by IRMM. A real-time PCR method for the quantification of AV43-6-G7 will be published after completion of its international validation by the European Reference Laboratory for GM food and feed (EURL-GMFF) on their homepage [12]. At IRMM, genomic DNA was extracted from 200 mg powder samples using a CTAB MicroSpin (S-400 HR, GE Healthcare Europe GmbH, Diegem, BE) DNA extraction method. Detection was done on an ABI 7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [13]. The real-time PCR measurements were calibrated with genomic DNA extracted from the pure AV43-6-G7 potato powder. After extraction the genomic DNA was diluted in a TE buffer solution (pH 8.3, 2 mmol/L TRIS and 0.2 mmol/L EDTA) and used to produce calibration curves for the transgenic and target taxon-specific gene. For the calibration curve of the transgenic gene the DNA was diluted between 4-times and 1000-times. For the calibration curve of the target taxon-specific gene the DNA was used undiluted and diluted up to 100-times. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of AV43-6-G7 and the obtained Ct-values. The diluted DNA was used to establish the calibration points for the transgenic gene. 3.3-times s of the lowest calibration point at which RSD was below 25 % was taken to calculate the LOD of the PCR method. The results of the quantification of AV43-6-G7 are shown in Table 2. Quantification of the mass fraction of

AV43-6-G7 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF431. However, as no independent calibration was carried out, the data displayed in Table 2 can be used for confirmation of the processing, but do not necessarily resemble the true value. It has to be noted that the calibrant used for the transgenic and the taxon-specific target is genomic DNA extracted from the pure AV43-6-G7 potato powder.

Table 2: Quantification of the AV43-6-G7 potato mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure AV43-6-G7 tuber powder for calibration

Candidate CRM	AV43-6-G7 potato mass fraction [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF431a	< 0.4 [1) 2)]	-
ERM-BF431b	880.4 [2)]	114.5
ERM-BF431c	9.3 [3)]	1.6
ERM-BF431d	34.0 [3)]	3.1
ERM-BF431e	87.2 [2)]	10.8

[1)] The obtained value is below the LOD determined during method validation (0.4 g/kg).

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

[3)] Mean for 3 samples (extraction replicates) from each of 12 random selected bottles (N = 12, n = 3), with each sample measured in three real-time PCR replicates.

4 Homogeneity

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

This homogeneity study was planned together with the measurements to control the gravimetric preparations and the short-term stability study (Section 3.5 and 5.1). As the measurement results were obtained under intermediate precision conditions on bottles randomly taken from the entire batch and analysed in a randomised order they were as well suited to investigate the homogeneity. Homogeneity of the pure non-GM material (blank) and the pure GM material is demonstrated by the test for the purity of the raw materials (Section 6.1).

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 of the material, within the stated uncertainties.

For the between-unit homogeneity test the number of selected bottles corresponds to approximately the cubic root of the total number of the produced bottles. Therefore, 12

bottles were selected for ERM-BF431c and d. In order to facilitate homogeneity studies and stability studies at the same time 15 bottles were selected for ERM-BF431e. A random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12 and 15 groups respectively (with similar number of bottles) and one bottle was randomly selected from each group. From each bottle, 3 independent samples were taken and analysed by real-time PCR. The measurements were performed under intermediate precision conditions due to the number of PCR plates required. Samples were analysed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. In the case of ERM-BF431e, data from the stability study at -70°C, 4°C and 18 °C could be pooled for determining the homogeneity, consequently 35 bottles were analysed. The results are shown in the figures in Annex A.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends in the analytical sequence were found for ERM-BF431c, d, and e at 99 % confidence level. The datasets were tested for consistency using Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. No outlying individual results and outlying unit means have been detected.

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

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

One has to bear in mind that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum heterogeneity that could be hidden by the intermediate precision of the method, was calculated as described by Linsinger *et al.* [14]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum heterogeneity that might be undetected by the given study setup.

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

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

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

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

MS_{within} mean square within a unit from an ANOVA

$MS_{between}$ mean squares between-unit from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

n mean number of replicates per unit

$v_{MSwithin}$ degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 3. In most cases, the uncertainty contribution for homogeneity was determined by the intermediate precision of the method.

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

CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF431c	17.6	3.6	5.5
ERM-BF431d	14.2	3.8	4.4
ERM-BF431e	16.1	n.c. [1)]	3.8

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

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

4.2 Within-unit homogeneity and minimum sample intake

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

5 Stability

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

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

The short-term stability study has been carried out using an isochronous design [15]. In that approach, samples of ERM-BF431e were stored for a certain time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible ("reference conditions"), effectively "freezing" the degradation status of the materials. At the end of the isochronous storage, the samples are analysed

simultaneously under conditions as repeatable as possible. Analysis of the material (after various exposure times and temperatures) under intermediate precision conditions greatly improves the sensitivity of the stability tests.

ERM-BF431 is a dried potato powder which has been processed similarly to other GMO CRM potato powders. As the water content of these powders and their particle size are similar, long-term stability data obtained in the frame of the stability monitoring of potato GMO CRMs were used for the estimation of the uncertainty instead of an individual long-term stability study. However, a 6 month long-term stability study was carried out to verify that stability of ERM-BF431 is similar to the stability established on the basis of earlier certified GMO potato CRMs.

5.1 Short-term stability study

For the short-term stability study, samples of ERM-BF431e have been stored at 4 °C, 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Five units per storage time were selected using a random stratified sampling scheme. From each unit, 3 samples were measured by real-time PCR. The measurements were performed under intermediate precision conditions using different PCR plates, and in a randomised manner to be able to separate a potential analytical drift from a trend over storage time.

The obtained data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test. One outlying result was found for the measurements at reference temperature. As no technical reason for the outlier could be found all data were retained for statistical analysis.

Furthermore, the data were plotted against storage time and regression lines of mass fraction versus time were calculated. The slope of the regression lines was then tested for statistical significance (loss/increase due to shipping conditions). The slopes of the regression lines were not significantly different from zero (99 % confidence level) for 4°C and 18 °C. A significant trend at 60 °C (99 % confidence level) was found.

The results of the measurements are shown in Annex B.

The material should therefore be shipped under cooled conditions.

5.2 Long-term stability study

Data from the stability-monitoring program for GMO CRMs were available. The GMO content in the potato powder CRMs (ERM-BF421b and ERM-BF430e) has been measured at 6 occasions over a period of 40 months. At each occasion, measurements were performed simultaneously on one PCR plate on units stored at +4 °C and -70 °C under repeatability conditions. In fact, each of these studies can be seen as a two-point isochronous study. The evaluation is based on the ratio of samples from +4 °C and -70 °C.

The results of the statistical evaluation of the stability monitoring showed that no outlying results were found and that no significant trend has been detected (99 % confidence level).

To verify that the data obtained from stability monitoring can be used to estimate the stability uncertainty contribution for ERM-BF431, an additional long-term stability study was organised for 6 months on ERM-BF431d. The data of this long-term stability study did not contradict with the data obtained from the stability monitoring.

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

The results of the measurements are shown in Annex C.

5.3 Estimation of uncertainties

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

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

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

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

RSD	relative standard deviation of all results of the stability study
x_i	result at time point *i*
$\bar{x}$	mean results for all time points
t_{tt}	chosen transport time (1 week at 18 ºC)
t_{sl}	chosen shelf life (24 months at 4 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. The uncertainty describes the possible change during a dispatch at 18 °C lasting for one week and was estimated to be 1.4 %.
- $u_{lts,rel}$, the stability during storage. The uncertainty contribution describes the possible degradation during 24 months storage at 4 °C and was estimated to be 2.8 %.

The uncertainty contributions arising from transport and storage have been considered in the combined uncertainty (Section 7). After the certification campaign, the material will be subjected to IRMM's regular stability monitoring programme to control its further stability.

6 Characterisation by gravimetric preparation

The material characterisation was based on a primary method of measurement, confirmed by an independent method. A primary method of measurement (also called "primary reference method" in the International Vocabulary of Metrology (VIM) [17]) is a method that does not require calibration with a standard of the same measurand and does not depend on a chemical reaction. Such methods are of highest metrological order and often yield results with very low uncertainties. However, it is nevertheless prudent to demonstrate absence of bias or gross errors by use of an independent method of lower metrological order.

For ERM-BF431 gravimetric mixing was chosen as the method of choice. The five candidate CRMs under the label code ERM-BF431 are potato powder materials processed from non-GM and GM tubers. While ERM-BF431a is prepared from the pure non-GM material (blank) and ERM-BF431b from the pure GM material, the other CRMs of the series are gravimetrically produced mixtures of the pure non-GM and GM tuber powders. ERM-BF431 is being certified for the mass fraction of AV43-6-G7 potato.

6.1 Purity of the starting materials

The purity of the GM and non-GM batches used for the processing of these powders was investigated in order to be able to calculate the certified value. Each individual potato used for the processing of the GM powder proved to be a starch-modified potato. Each individual potato used for the processing of the non-GM powder proved to be a non-starch-modified potato (Section 3.1). As no evidence for a contamination was found in both starting materials, the certified value for ERM-BF431a and ERM-BF431b is therefore based on the fact that the material has a purity of 100 %. Also for the calculation of the certified mass fraction of AV43-6-G7 in the powder mixtures 100 % purity was used.

6.2 Mass fractions and their uncertainties

The certified mass values are based on the mass fractions of dry-mixed GM and non-GM powders, corrected for their water mass fractions, and taking into account the powder's purity with regard to the AV43-6-G7 event. The values were calculated according to the following formulas:

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

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

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

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

In Table 4, the data supporting the calculation of the mass fractions of AV43-6-G7 potato are summarised.

Table 4: Subsequent mixing of GM AV43-6-G7 potato tuber powder (ERM-BF431b) with non-GM powder (ERM-BF431a) to prepare the ERM-BF431c, d, and e materials

CRM	GM powder			Non-GM powder [1)]	Mixtures
	Mass fraction of GM powder [g/kg]	Water mass fraction ± *U* (*k* = 2) [g/kg]	Mass [g]	Mass [g]	Resulting mass fraction of GM powder [g/kg]
ERM-BF431e	1000.0 [2)]	17.9 ± 4.6	393.6	3600.6	99.3
ERM-BF431d	100.0 [3)]	23.6 ± 6.0	1595.6	2399.4	39.7
ERM-BF431c	100.0 [4)]	23.6 ± 6.0	397.2	3599.0	9.9

[1)] The non-GM powder (ERM-BF431a) used for the gravimetric preparations had a water mass fraction of 25.8 ± 6.6 g/kg (*U, k* = 2) and was considered to be free of AV43-6-G7 potato.

[2)] For the preparation of ERM-BF431e the pure GM powder (ERM-BF431b) was used.

[3)] For the preparation of ERM-BF431d the 100 g/kg GM powder (ERM-BF431e) was used.

[4)] For the preparation of ERM-BF431c the 100 g/kg GM powder (ERM-BF431e) was used.

The uncertainties on the certified mass fractions (u_{char}) of AV43-6-G7 potato is composed of several contributions, i.e. the uncertainty on the mass determination ($u_{\text{char,1}}$) and the uncertainty on the water mass fraction analysis ($u_{\text{char,2}}$). No uncertainty contribution was taken into consideration for the purity determination of the non-GM and GM base powders (Table 5).

Table 5: Characterisation related uncertainty budgets for the mass fractions of AV43-6-G7 potato in ERM-BF431

CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]		Combined uncertainty u_{char} [g/kg]
		$u_{char,1}$ [1)]	$u_{char,2}$ [2)]	
ERM-BF431a	**0**	n.a.	n.a.	**n.a**
ERM-BF431b	**1000**	n.a.	n.a.	**n.a.**
ERM-BF431c	**10**	0.1276	0.0562	**0.1394**
ERM-BF431d	**40**	0.3758	0.2259	**0.4384**
ERM-BF431e	**100**	0.9115	0.4618	**1.0218**

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

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

6.3 Confirmation measurements

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

7 Value Assignment

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

The certified values are based on the purity of the raw material used to process ERM-BF431a and ERM-BF431b and for the mixtures on the masses of dried powders of GM tubers and non-genetically modified tubers used in the gravimetrical preparation. The masses of the powders were corrected for their respective water mass fractions during the preparation of the materials (Table 4).

The assigned uncertainty is calculated from uncertainties related to characterisation, u_{char} (Section 6.2), potential between-unit heterogeneity, u_{bb} (Section 4.1), short-term stability, u_{sts} (Section 5.3) and long-term storage, u_{lts} (Section 5.3). These different contributions were combined to estimate the expanded uncertainty of the certified value (U_{CRM}) with a coverage factor k as

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

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

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

Table 6: Certified values and their uncertainties for ERM-BF431

CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{lts} [g/kg]	U_{CRM} [g/kg] [1)]
BF431a	0	n.a.	n.a	n.a	n.a	-
BF431b	1000	n.a.	n.a	n.a	n.a	-
BF431c	9.9	0.1394	0.5426	0.1381	0.2763	1.3
BF431d	40	0.4384	1.7444	0.5550	1.1101	5
BF431e	99	1.0218	3.7720	1.3897	2.7794	10

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

As it is not known how the certified GM powder mass fractions are related to the corresponding transgenic and target taxon-specific DNA copy number ratio, the user is reminded that IRMM only certifies these materials for their mass fraction of AV43-6-G7. Additionally, one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements on unknown samples as DNA- and/or protein-based quantification of GMOs may vary with the particular matrix and the variety tested.

8 Metrological traceability and commutability

8.1 Metrological traceability

Quantity value

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

8.2 Commutability

Many measurement procedures include one or more steps, which are selecting specific (or specific groups) of analytes from the sample for the subsequent steps of the whole measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all the analytically relevant

properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is nowadays summarised in a concept called 'commutability of a reference material'. There are various definitions expressing this concept. For instance, the CSLI Guideline C-53A [18] recommends the use of the following definition for the term *commutability*:

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

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

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

9 Instructions for use

9.1 Storage conditions

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

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

9.2 Safety and protection for the environment

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

9.3 Minimum sample intake

The minimum sample intake is 200 mg.

9.4 Use of the certified value

The main purpose of these materials is the use for calibration or quality control of AV43-6-G7 potato detection methods. As any reference material, they can also be used for control charts or validation studies.

Use as a calibrant

If this matrix material is used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty. Furthermore it should be noted that using the same material for calibration and quality control limits the control possibilities as calibrant and quality control material are based on the same raw materials. If unavoidable, it is recommended to use different concentration levels of ERM-BF431 for calibration and for quality control.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [19].

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

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

Use in quality control charts

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

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Annexes

Annex A: Results of the homogeneity measurements

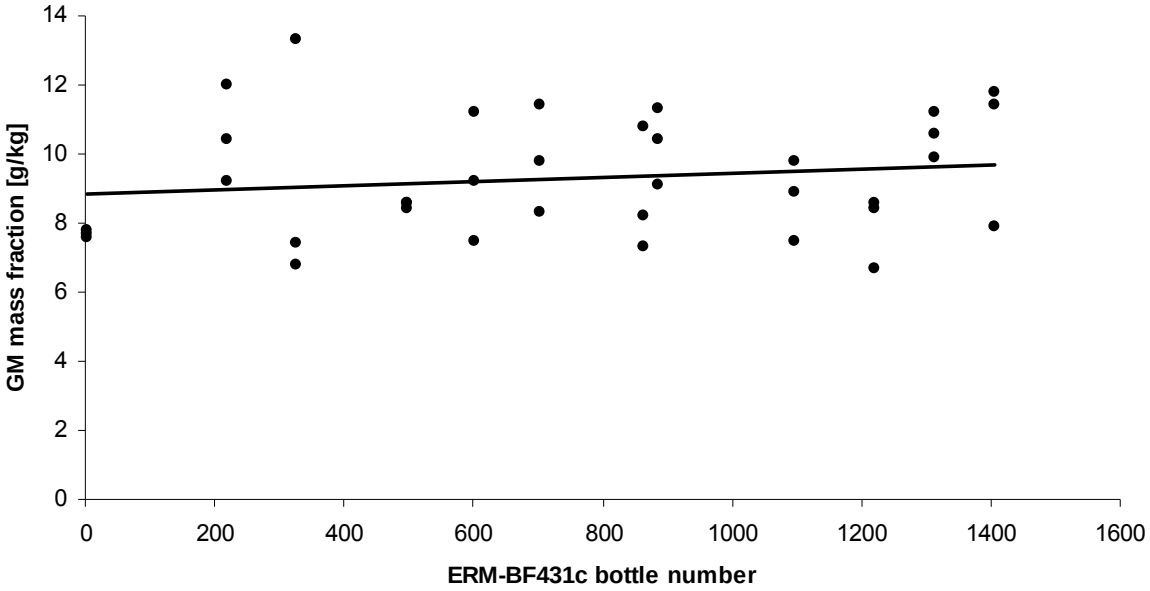

Figure A1: Real-time PCR measurement results obtained for ERM-BF431c (*N* = 12, *n* = 3, measured in triplicate). The linear regression for all data points is given.

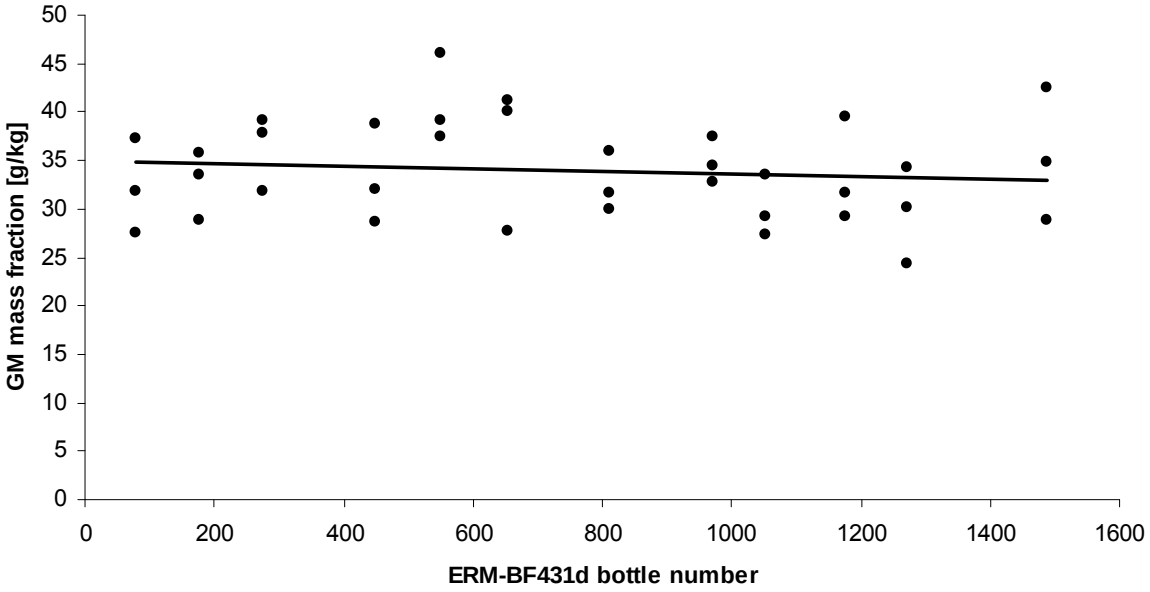

Figure A2: Real-time PCR measurement results obtained for ERM-BF431d (*N* = 12, *n* = 3, measured in triplicate). The linear regression for all data points is given.

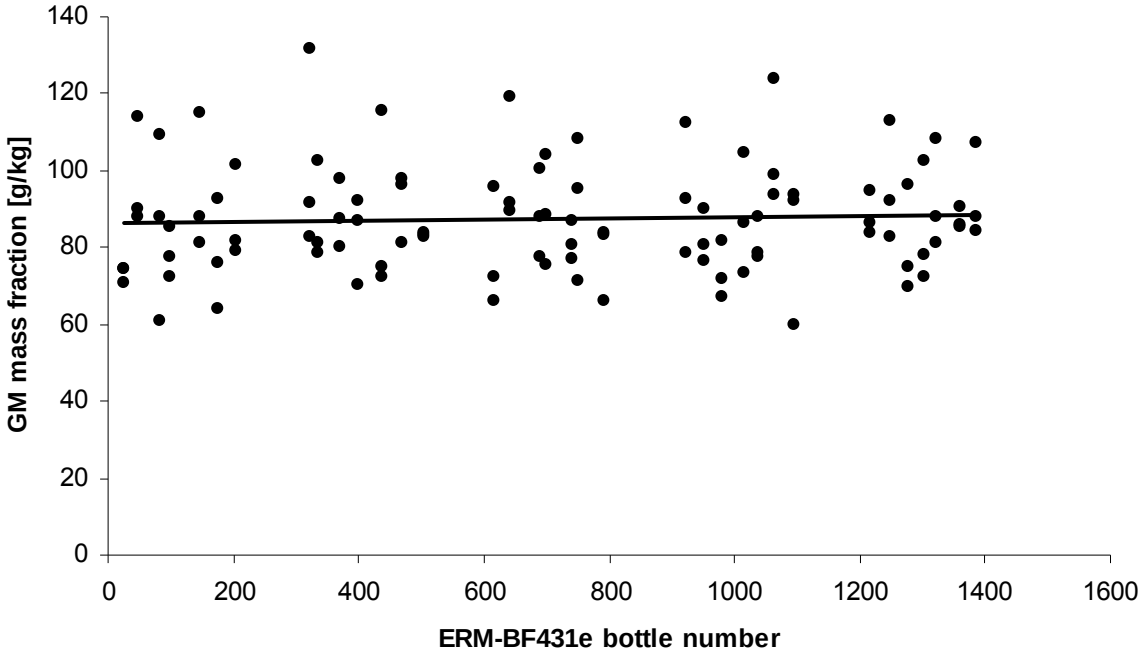

Figure A3: Real-time PCR measurement results obtained for ERM-BF431e (*N* = 35, *n* = 3, measured in triplicate). The linear regression for all data points is given.

Annex B: Results of the short-term stability measurements

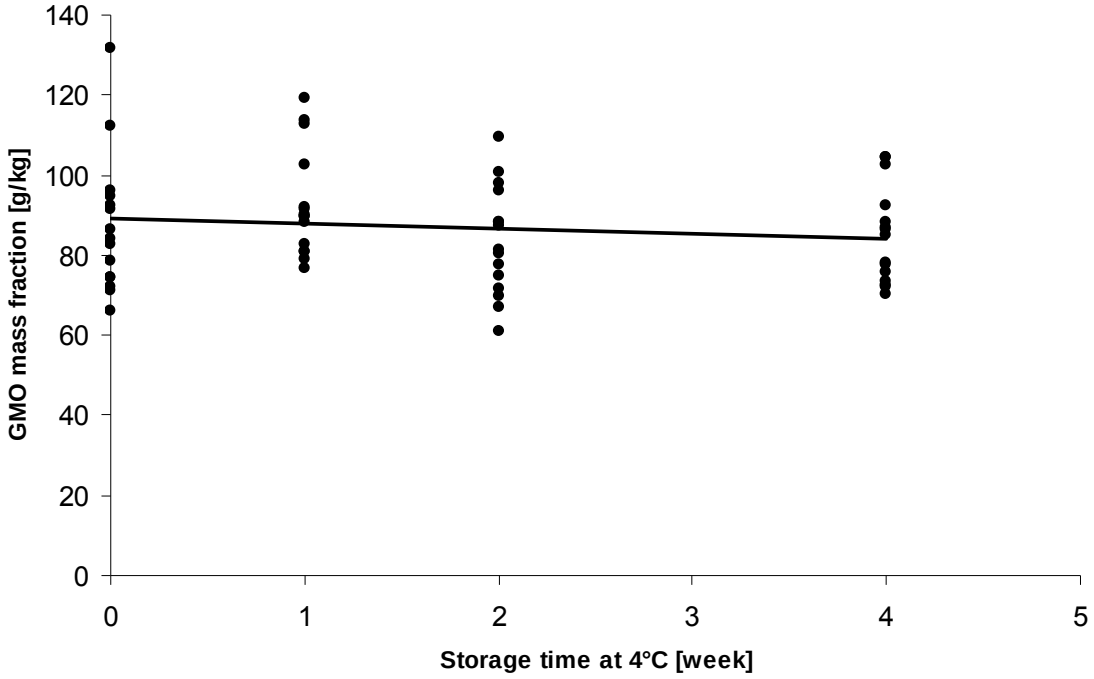

Figure B1: Real-time PCR measurement results obtained for ERM-BF431e during short-term stability testing (*N* = 5, *n* = 3, measured in triplicate) at 4°C. The linear regression for all data points is given.

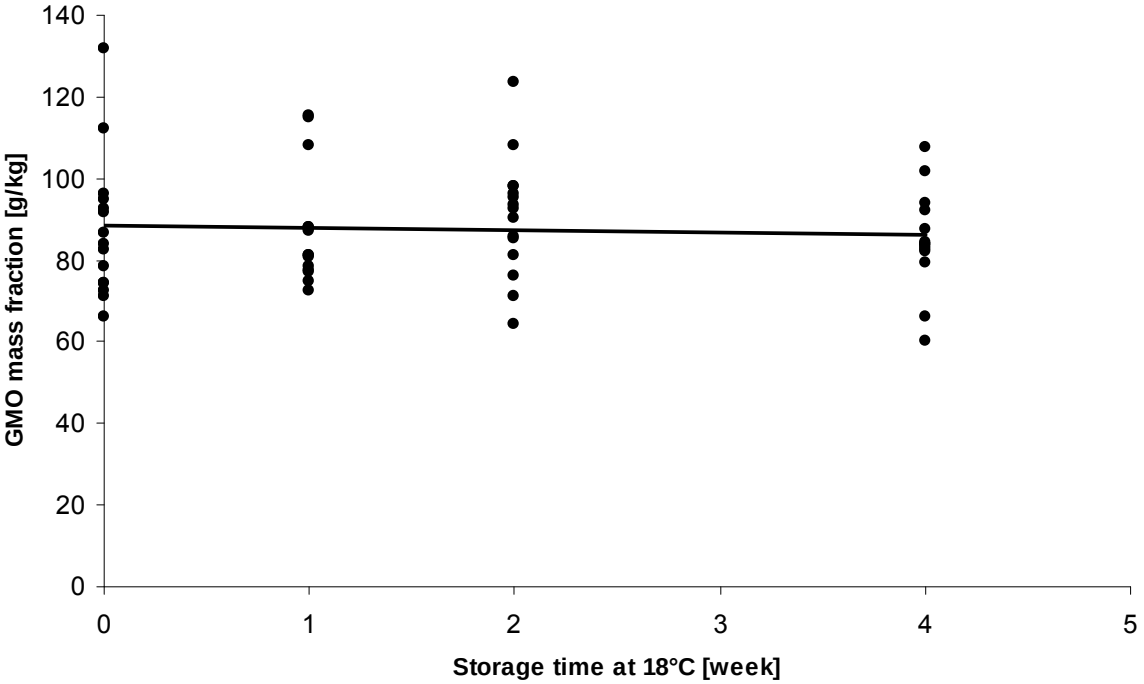

Figure B2: Real-time PCR measurement results obtained for ERM-BF431e during short-term stability testing (*N* = 5, *n* = 3, measured in triplicate) at 18°C. The linear regression for all data points is given.

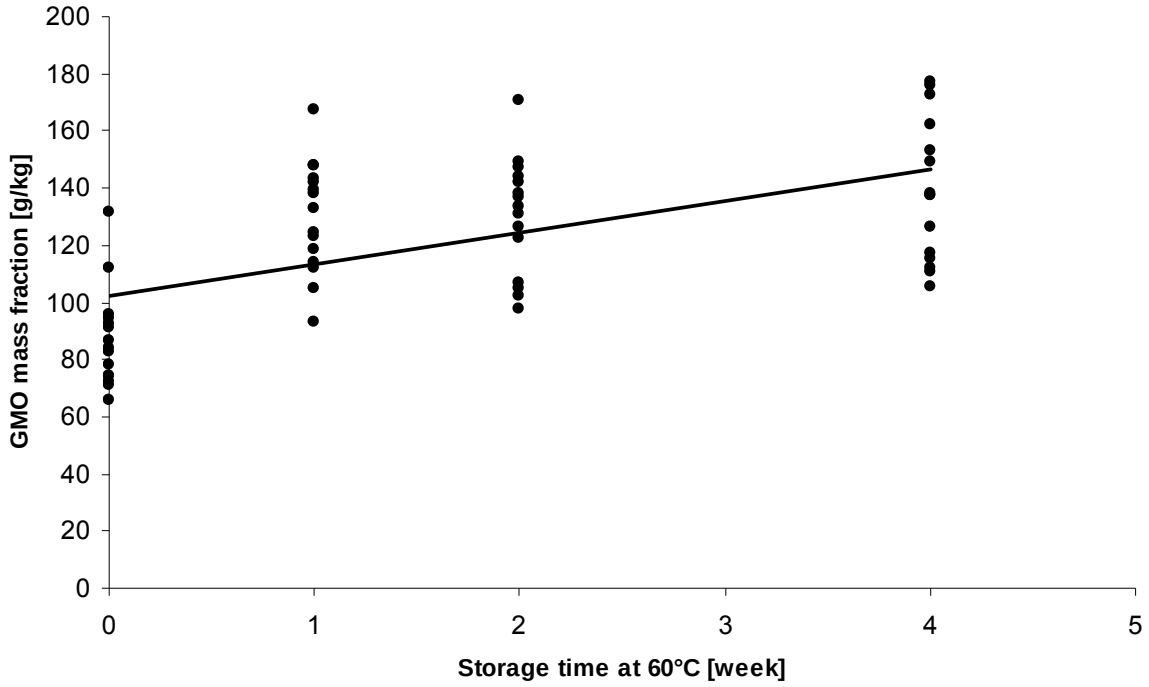

Figure B3: Real-time PCR measurement results obtained for ERM-BF431e during short-term stability testing (*N* = 5, *n* = 3, measured in triplicate) at 60°C. The linear regression for all data points is given.

Annex C: Results of the long-term stability measurements

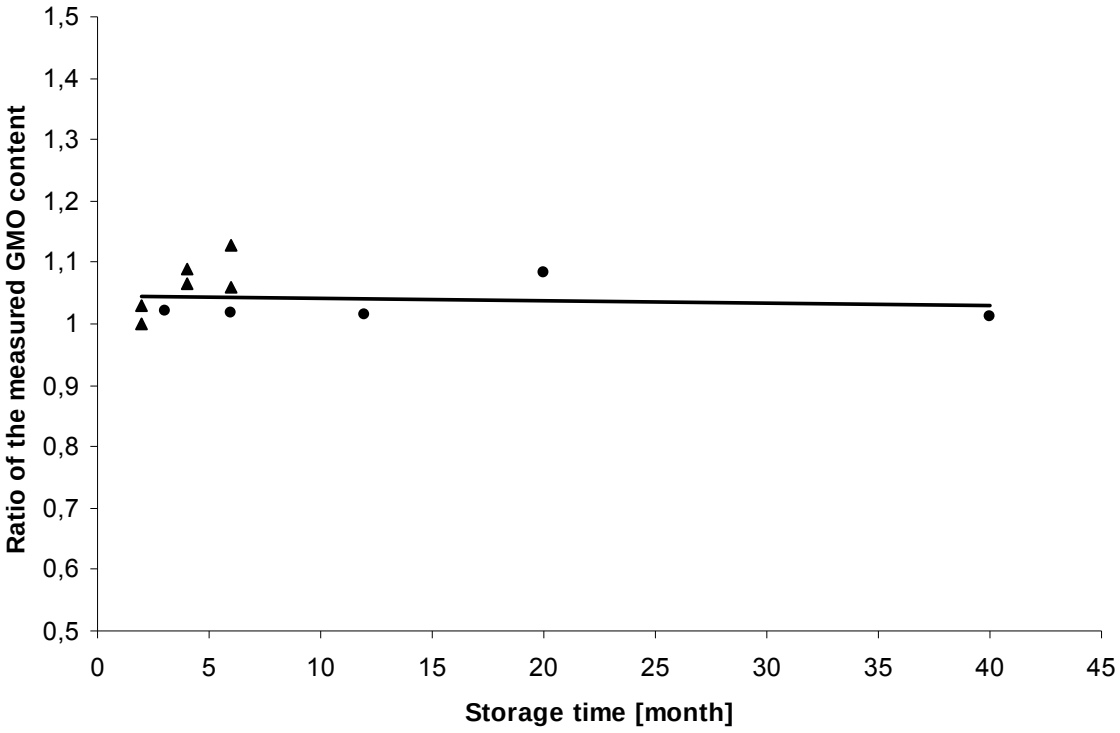

Figure C1: Real-time PCR measurement results obtained for ERM-BF421b and BF430e, during post certification monitoring for the measured ratio of 4C°/-70°C (●) and for ERM-BF431d during the 6 month long-term stability study for the measured ratios of 4C°/-70°C and 18C°/-70°C (▲).

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Abstract
This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF431a, b, c, d and e, matrix materials certified for their AV43-6-G7 mass fractions. The material has been produced following ISO Guide 34:2009 [1].
Genetically modified (GM) tubers of the potato event AV43-6-G7 and of a non-GM potato variety were dried and ground to obtain GM and non-GM potato powders. Beside these two pure materials gravimetric mixtures of non-GM and GM potato powder were prepared by dry-mixing.
Between unit-heterogeneity has been quantified and stability during dispatch and storage have been assessed in accordance with ISO Guide 35:2006 [2].
The certified value was obtained from the gravimetric preparations, taking into account the purity of the base materials and their water mass fraction. The certified values were confirmed by event-specific real-time PCR as independent confirmation method (measurements within the scope of accreditation to ISO/IEC 17025:2005 [3]).
Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible heterogeneity, instability, and characterisation.
The materials are intended for the calibration or quality control of AV43-6-G7 potato identification and quantification methods. As any reference material, they can also be used for control charts or validation studies. The CRMs are available in glass vials containing at least 1 g of dried potato tuber powder and closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.
The CRM has been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

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