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CERTIFICATION REPORT

The certification of different mass fractions of the GM event 73496 in rapeseed powder

Certified Reference Materials ERM®-BF434a, ERM®-BF434b, ERM®-BF434c, ERM®-BF434d and ERM®-BF434e

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The certification of different mass fractions of the GM event 73496 in rapeseed powder

Certified Reference Materials ERM®-BF434a, ERM®-BF434b, ERM®-BF434c, ERM®-BF434d and ERM®-BF434e

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Summary

This report describes the production of the set of Certified Reference Materials (CRMs) ERM-BF434a, b, c, d and e. These are matrix materials certified for their 73496 rapeseed mass fractions. Materials have been produced following ISO Guide 34:2009 [1].

Genetically modified (GM) rapeseeds of the event 73496 and of a non-GM rapeseed variety were ground to obtain GM and non-GM starting materials. Gravimetric mixtures of non-GM and GM rapeseed powder were prepared by dry-mixing.

The between unit-heterogeneity has been quantified and stability during dispatch (short-term stability) and stability during storage (long-term stability) 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 seeds used for the processing of the materials and their respective water mass fraction. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements within the scope of accreditation to ISO/IEC 17025:2005 [3]).

Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties related to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).

The materials are intended for the calibration or quality control of 73496 rapeseed identification and quantification measurements expressed in mass fractions. 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 rapeseed powder closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.

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

The following values were assigned:

	73496 mass fraction [1)]	
	Certified value [2)] [g/kg]	Uncertainty [3)] [g/kg]
ERM-BF434a	< 0.04	-
ERM-BF434b	> 988	-
ERM-BF434c	1.00	0.15
ERM-BF434d	10.0	1.4
ERM-BF434e	100	12

1) Genetically modified 73496 rapeseed with the unique identifier DP-Ø73496-4.

2) Mass fraction of 73496 rapeseed based on the masses of powder of genetically modified 73496 rapeseed and powder of non-modified rapeseed 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.

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Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression y = a + *b*x
CLSI	Clinical and Laboratory Standards Institute
CRM	Certified reference material
DNA	Deoxyribonucleic acid
EC	European Commission
ERM®	Trademark of European Reference Materials
EU	European Union
EU-RL GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurements [4]
EDTA	Ethylenediamintetraacetic acid
IEC	International Electrotechnical Commission
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
JRC	Joint Research Centre of the EC
k	Coverage factor
LOD	Limit of detection
n	Number of replicates per unit
N	Number of samples (units) analysed
PCR	Polymerase chain reaction
PSA	Particle size analysis
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation; an additional index "rel" is added as appropriate
t	Time
t_i	Time point for each replicate
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
TE	Tris-EDTA
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
V-KFT	Volumetric Karl Fischer titration
$\overline{x}$	Arithmetic mean

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 and need to be labelled if they contain more than 0.9 % of GMOs [5]. This labelling threshold is applicable for adventitious presence of GMOs, while GMOs added on purpose need to be labelled independent from a threshold. Additionally feed may contain 0.1 mass percent of a GM event for which an authorisation process is pending or the authorisation in the EU has expired [6]. In general, this threshold demands on the development and validation of reliable methods for GMO quantification, and the production of reference materials for calibration or quality control of these methods.

DuPont Pioneer (Iowa, US) has developed the genetically modified (GM) 73496 rapeseed event with the unique identifier code DP-Ø73496-4 following Commission Regulation (EC) No 65/2004 [7]. In 2011 the Institute for Reference Materials and Measurements (IRMM, Geel, BE) was asked to produce a reference material for the quantification of 73496 rapeseed. The intended effect of the modification is to confer tolerance to the herbicide glyphosate. To accomplish this objective, Pioneer introduced the *gat4621* gene that encodes the glyphosate N-acetyltransferase (GAT4621) protein, which confers tolerance to glyphosate. The GAT4621 protein renders the rapeseed tolerant to glyphosate through acetylation of glyphosate to the non-phytotoxic N-acetylglyphosate [8]. The Certified Reference Material (CRM) produced by IRMM has been named ERM-BF434 and is composed of a set of five CRMs with different mass fractions of 73496 rapeseed.

1.2 Choice of the material

The set of CRMs ERM-BF434 was produced from milled seeds of GM and non-GM rapeseed. Seeds were selected as a raw material source as they allowed testing of the genetic purity with a respect to the event 73496.

1.3 Design of the project

Besides the pure non-GM material ERM-BF434a and the pure GM material ERM-BF434b, all gravimetric mixtures of non-GM and GM rapeseed powder were prepared by dry-mixing. The first material ERM-BF434e was prepared by mixing pure non-GM with GM rapeseed powder. ERM-BF434d was prepared by further dilution of ERM-BF434e and ERM-BF434c was prepared by further dilution of ERM-BF434d, in both cases with non-GM rapeseed powder.

The different mass fractions of ERM-BF434 were certified using a gravimetric approach.

2 Participants

2.1 Raw material provider

DuPont Pioneer (Iowa, US).

2.2 Project management and evaluation

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

2.3 Processing and analytical measurements

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 GM quantification, BELAC No. 268-TEST)

DuPont Pioneer (Iowa, US)
(in accordance with the requirements of GLP Program, United States Environmental Protection Agency)

3 Material processing and process control

3.1 Origin of the raw materials

For the preparation of the CRMs, Pioneer, supplied non-GM rapeseed powder and 73496 rapeseed powder to IRMM. The materials were shipped at ambient temperature and after arrival, they were stored at (4 ± 3) °C in dark until used for processing.

The purity of the delivered GM and non-GM powders had been measured by Pioneer. For the GM rapeseed, the purity was tested using the real-time qualitative event-specific PCR assay on DNA extracted from individual single-seed. One out of 399 seeds in the GM material was detected to be negative for the event 73496. Taking the 95 % confidence interval into account this leads to a purity level of the GM seed lots > 98.81 %. The calculated lot purity of the GM seed batch was taken into account for the estimation of the uncertainties associated to the certified values of the reference materials (Section 6.2).

For the purity of the non-GM rapeseed, Pioneer used a same event-specific PCR assay capable of detecting one positive seed in a pool of 1600 seeds. The batch was divided in four pools each containing 1600 seeds. Five DNA extractions were prepared from each of the four pools; real-time PCR was performed in triplicate on each of the 20 DNA extractions. Impurity of the material with respect to 73496 rapeseed was not detected.

3.2 Processing

The first processing steps of the non-GM and 73496 rapeseed raw materials were carried out by Pioneer using two models of coffee grinders (DeLonghi KG94 and DCG-39). Each individual coffee grinder was designated to be used only on either the non-GM or GM rapeseed to assure that no cross-contamination took place. Non-GM processing occurred in an isolated room, well apart from GM rapeseed processing. Both powders were ground in batches of approximately 25 g. For the further processing of ERM-BF434 at IRMM, about 30 kg of non-GM rapeseed powder and 8 kg of 73496 rapeseed powder were shipped to IRMM.

After the reception at IRMM, the non-GM and GM powders were mixed separately in DynaMIX CM200 (WAB, Basel, CH) for half an hour and the water mass fraction was determined by volumetric Karl Fischer titration (V-KFT). The non-GM powder had a remaining residual water mass fraction of about 72.1 ± 1.8 g/kg and the GM powder had a remaining residual water mass fraction of about 66.5 ± 0.6 g/kg.

The GM and non-GM starting materials were processed separately. Cross-contamination and contamination with foreign DNA were avoided applying systematic cleaning, clean laboratory clothing and measures to prevent cross-contamination by air. All contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals) prior to exposure to the materials. An in-house validation study had proven beforehand that the solution degraded DNA effectively under the given conditions. If required, the starting materials were stored for short time periods in closed plastic containers.

In order to produce starting materials that could be used for production of the reference materials, the mixed non-GM and GM 73496 rapeseed powders were sieved separately over a 500 µm stainless steel mesh on a sieving machine (Russel Finex, London, UK). The coarse fractions were placed in liquid nitrogen and milled with a previously cooled mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE) at temperature below -90 °C. The feeding speed of the mill was adjusted to ensure most efficient milling with respect to the particle size obtained.

After the second sieving step, a coarse fraction of 40 g from the GM powder did not pass the 500 µm mesh and was discarded. After the sieved powder was added to the rest of the powder that had passed through 500 µm stainless steel mesh, it was mixed using a DynaMIX CM200 for half an hour to improve equal distribution of the different types of rapeseed tissues because the milling and sieving processes caused the separation of the different tissues from each other.

In order to facilitate the dry mixing, the water content was reduced. The powders were dried for 5.5 hours under vacuum in a dryer (FD Epsilon 2-10D, Osterode, DE) at 20 °C. For the non-GM and GM powders, a residual water mass fraction (N = 1, n = 3) of 31.2 ± 0.9 g/kg and 41.5 ± 1.2 g/kg respectively was measured by Volumetric Karl Fischer titration (V-KFT, 758 KFD Titrino, Metrohm, Herisau, CH) with the expanded uncertainty calculated with k = 2. The particle volumes for both powders were measured by laser diffraction (Sympatec, Clausthal-Zellerfeld, DE) and compared (Figure 1). The mean particle diameters (N = 1, n = 5), calculated by the PSA software of the instrument, were 212 µm (s = 8 µm) for the non-GM powder and 201 µm (s = 9 µm) for the GM powder. It is important to understand that the cumulative volume distribution of particles derived from laser light scattering data is based on their equivalent spherical diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. Since most particles are presumably not perfectly spherical, the calculated volume of the particles based on their diameter is, therefore, overestimating the mean particle size. It has been concluded that the particle volume fractions of the non-GM and GM starting materials were sufficiently similar to allow the processing of mixtures without introducing a bias.

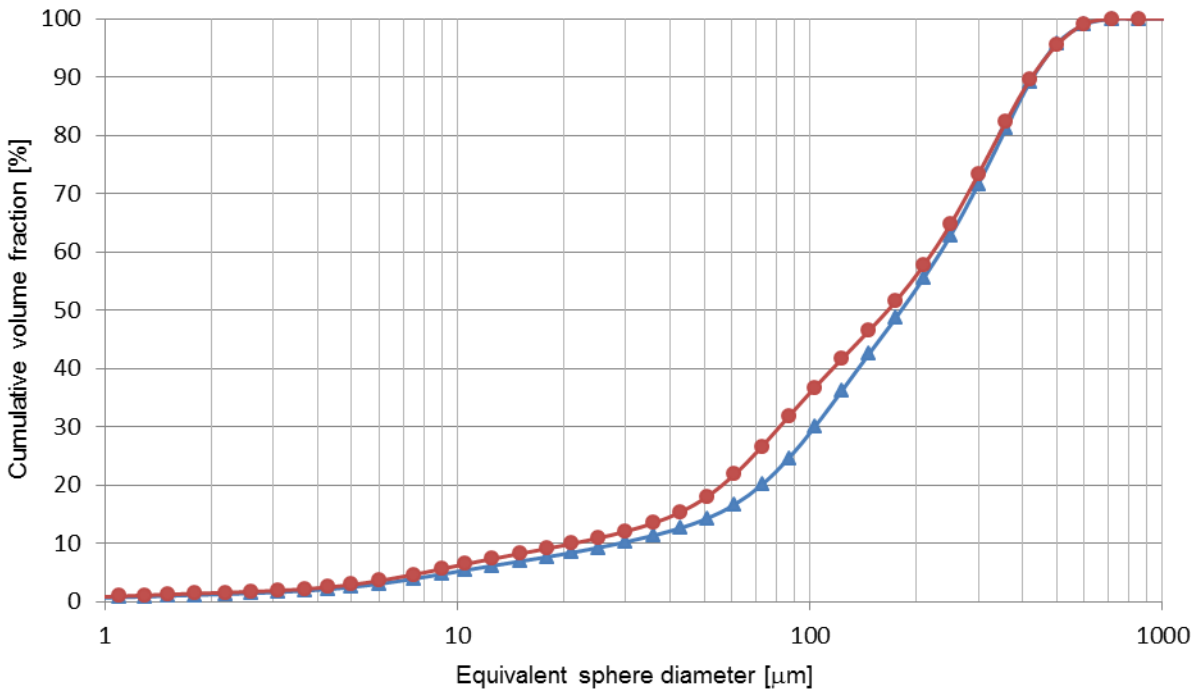

Figure 1: Cumulative representation of particle volume fractions in the non-GM powder (▲) and GM powder (●) analysed by laser diffraction (N = 1, n = 5). The total volume is set as 100 %.

The ground materials were used to produce a blank material for 73496 (non-GM rapeseed powder), a pure GM rapeseed powder and three mixtures containing different mass fractions of 73496 rapeseed powder in non-GM rapeseed powder at nominal mass fraction levels of 1, 10 and 100 g/kg. The term "nominal" is used to discriminate between the value targeted in the processing and the certified value assigned after completion of the certification process.

All these materials, including the blank powder and the pure GM 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 was verified before use. Portions were weighed into a container and mixed for 30 min using a DynaMIX 200C. The blank material was processed first, followed by the mixtures. For the preparation of the mixtures, the masses of the non-GM and GM powders were corrected for their respective water mass fractions. The masses which are theoretically needed to reach a certain nominal mass fraction were calculated. The material having a nominal mass fraction of 100 g/kg of 73496 rapeseed was produced by mixing pure GM with pure non-GM ground starting materials. The material having a nominal mass fraction of 10 g/kg 73496 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 thereafter produced by further dilution of the 10 g/kg GM powder with pure non-GM powder. At each mixing step, the water mass fraction of the mixed materials was taken into account (Table 5). For the certification process, the gravimetric preparation was the basis for the calculation of the mass fraction of the powders (Section 6).

All produced powders were filled in 10 mL brown glass vials using an automatic filling device. The first 30 bottles of each batch were discarded as an additional precaution against carry-over contamination. Lyophilisation inserts were automatically placed in the bottle necks. Before final closure of the vials, air was evacuated in a freeze-dryer and replaced by argon. The vials were finally closed inside the freeze-dryer with the help of a hydraulic device and then sealed with aluminium caps to prevent accidental opening during storage and transport. Colour-coded caps were used for easy identification of the different mass fraction levels of 73496 rapeseed: nominal 0 g/kg = silver (BF434a), nominal 1000 g/kg = black (BF434b), nominal 1 g/kg = yellow (BF434c), nominal 10 g/kg = red (BF434d) and nominal 100 g/kg = brown (BF434e), consistent with the cap colours of previous IRMM CRMs for GMOs. Each of the vials was identified by a numbered label indicating the ERM code and the unit number. Following the inventory and the selection of vials for future analysis according to a random stratified sampling scheme, the bottles were brought to a storage room for long-term storage in the dark at (4 ± 3) °C.

3.3 Process control

The residual mass fraction of water in five 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-BF434 determined by V-KFT (N = 5, n = 2)

Candidate CRM	Water mass fraction [g/kg]	
	$\bar{x}$	s
ERM-BF434a	21.9	1.7
ERM-BF434b	25.9	1.5
ERM-BF434c	20.0	2.0
ERM-BF434d	20.2	1.4
ERM-BF434e	14.4	1.0

Five randomly selected bottles from each of the powder materials were analysed twice for their particle volume distribution (N = 5, n = 2) by laser diffraction (Sympatec, Clausthal-Zellerfeld, DE). The powders have a particle diameter below 1220 µm (Figure 2). The mean particle diameters (N = 5, n = 2), calculated by the PSA software of the instrument, were 221 µm (s = 14 µm), 235 µm (s = 13 µm), 212 µm (s = 8 µm), 190 µm (s = 31 µm) and 205 µm (s = 10 µm) for ERM-BF434a, b, c, d and e respectively.

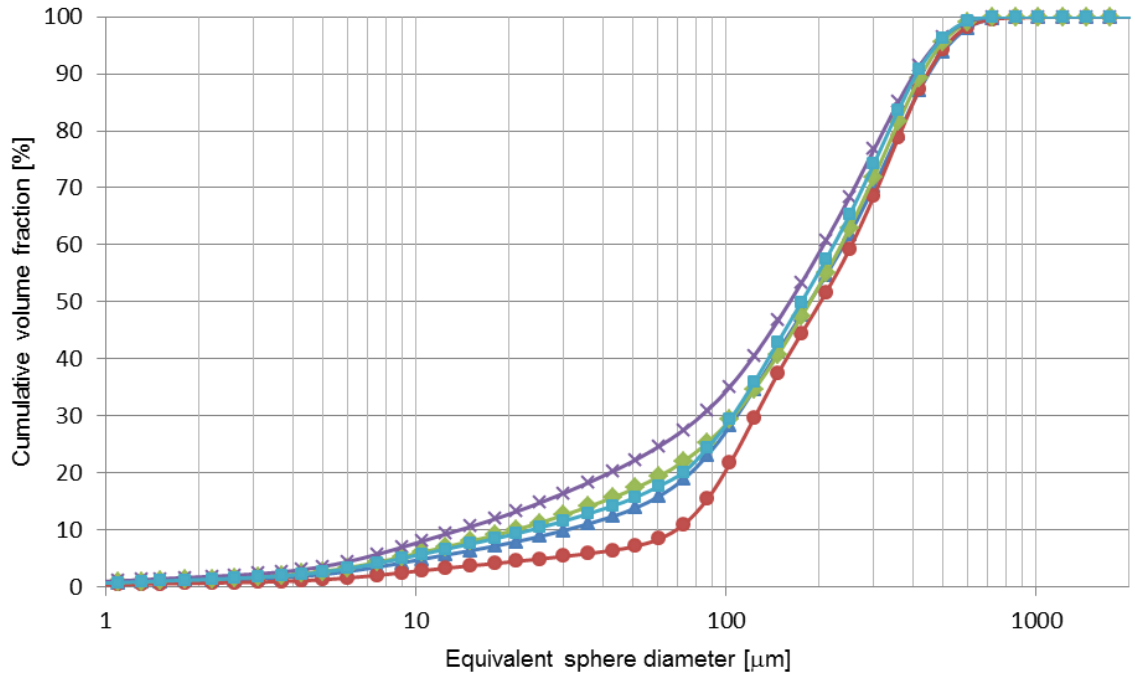

Figure 2: Cumulative representation of particle volume fractions in ERM-BF434a (▲), ERM-BF434b (●), ERM-BF434c (◆), ERM-BF434d (×) and ERM-BF434e (■), analysed by laser diffraction (N = 5, n = 2). The total volume was set as 100 %.

3.4 DNA content of the starting materials

Three of the described CRMs are mixtures of GM and non-GM rapeseed 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. Consequently, any difference in the extractable DNA mass fraction in the non-GM and GM starting materials would lead to a shift of the measurement results obtained with e.g. real-time PCR.

The mass of DNA in both materials was estimated using a slight modification of the classical fractionation method developed initially by Ogur and Rosen [9]. A sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds followed by an acidic extraction of DNA 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 a colorimetric reaction using diphenylamine. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [9, 10]. The absorbance was measured at 600 nm wavelength with a spectrophotomer.

The extractable DNA mass fraction of the two materials was calculated as:

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

The ratio of the DNA mass extractable from 100 mg of GM and non-GM rapeseed powder was found to be (1.18 ± 0.03), (N = 9 with an expanded uncertainty, k = 2). A t-test with 99 % confidence level demonstrated that a significant difference exists from 1. Consequently the GM mass fractions of ERM-BF434 prepared gravimetrically are not equivalent to the ratio of DNA copy number measured.

The DNA integrity was checked by gel electrophoresis. DNA was extracted from 200 mg samples of the processed powder materials ERM-BF434a, ERM-BF434b, ERM-BF434c, ERM-BF434d and ERM-BF434e. After extraction, approximately 1.0 µg DNA per sample was loaded on an agarose gel (mass concentration of 7.5 agarose g/L). Staining of the DNA was done with an ethidium bromide solution (0.5 mg/L). None of the samples showed DNA degradation (data not shown).

3.5 Confirmation measurements

As a control for the gravimetric preparations, the mass fraction of 73496 rapeseed in all five CRMs was confirmed by a real-time PCR method provided by Pioneer and delivered under confidentiality agreement to IRMM. This method targets the transgenic DNA insertion in this rapeseed event and will be published after validation by the European Reference Laboratory for GM Food and Feed (EU-RL GMFF) on their home page [11].

The real-time PCR test was calibrated with genomic DNA extracted from the pure 73496 rapeseed powder. At IRMM, the genomic DNA was extracted by an in-house validated DNA extraction method using 200 mg powder samples. After extraction the DNA was diluted in a TE buffer solution (pH 8.0, 1 mmol/L TRIS and 0.01 mmol/L EDTA) to the final concentration of 40 ng/µL and used to produce calibration curves for the rapeseed-specific gene and the transgene as well. Detection was done on an ABI 7900 HT real-time PCR instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, US) [12]. For the calibration curve for the measurement of the rapeseed specific gene FatA(A), the DNA was used undiluted with concentration of 40 ng/µL and diluted up to 200-fold. For the calibration curve of the transgene the DNA was diluted from 4-fold to 10000-fold. The LOD of the PCR method was taken as 3.3-fold s of the lowest calibration point at which RSD was below 25 %. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of 73496 and the obtained quantification cycle (Cq) values. The results of the quantification of 73496 are shown in

Table 2. Quantification of the mass fraction of 73496 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF434. 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 represent the true value.

Table 2: Quantification of the 73496 rapeseed mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure 73496 rapeseed powder for calibration

Candidate CRM	73496 rapeseed mass fraction [g/kg]	U (k = 2) [g/kg]
ERM-BF434a	< 0.04 [1) 2)]	-
ERM-BF434b	890.3 [1)]	89.6
ERM-BF434c	1.0 [3)]	0.1
ERM-BF434d	10.6 [4)]	1.1
ERM-BF434e	102.5 [1)]	10.3

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

2) The obtained value is below the LOD determined during method validation (0.04 g/kg).

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

4) Mean of 5 extraction replicates from each of 5 randomly selected bottles (N = 5, n = 5), 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 necessarily relevant if the variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

This homogeneity study was planned together with the measurements to control the gravimetric preparations and the short-term stability study (Sections 3.5 and 5.1). As the measurement results were obtained under intermediate precision conditions on bottles randomly taken from the entire batch and analysed in a randomised order, they were as well suited to investigate the homogeneity. The intermediate precision of the in-house validated method was sufficiently good to analyse two replicates of each sample of the reference materials and obtain an acceptable u^*_{bb}. An exception was only ERM-BF434c where three replicates of each sample were needed to be analysed. Homogeneity of the blank material is demonstrated by the test for the purity of the non-GM starting material (Section 3.1). No specific homogeneity study was done for ERM-BF434a and ERM-BF434b.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the candidate CRMs are valid for all bottles containing 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-BF434c and ERM-BF434d. In order to facilitate homogeneity studies and short-term stability study, 15 bottles were selected for ERM-BF434e. For each CRM a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12 and 15 groups respectively (with similar number of bottles) and one bottle was randomly selected from each group. From each bottle of ERM-BF434d and ERM-BF434e, 2 independent samples were taken and analysed by real-time PCR. An exception was ERM-BF434c, where three replicates of each sample were taken. Due to the number of PCR plates required, the measurements were performed under intermediate precision conditions. Samples were analysed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. The results are shown in the figures in Annex A.

Regression analyses were performed to evaluate potential trends in the filling sequence. No significant trends were observed on a 99 % confidence level.

Furthermore, regression analyses were performed to evaluate potential trends in the analytical sequence. As this concerns an attribute of the analytical system that can be corrected for, a less stringent confidence level of 95 % was used. Statistically significant trend was detected for ERM-BF434d and ERM-BF434e, pointing to a drift of the signal in the analytical system. The correction of biases was found to combine the smallest uncertainty with the highest probability to cover the true value. As the analytical sequence and the unit numbers were not correlated, trends significant on at least a 95% confidence level were corrected as shown below [13].

$$\text{corrected result} = \text{measured result} - b \cdot i$$

b = slope of the linear regression
i = position of the result in the analytical sequence

The trend-corrected dataset was tested for consistency using Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. No trends were present in the analytical sequence of ERM-BF434d and ERM-BF434e.

The datasets were tested for consistency using single and double Grubbs outlier tests on a confidence level of 99 % on the individual results and the unit means. The trend-corrected datasets showed no outliers at the level of unit means, but in ERM-BF434c two outlying individual results were detected. As no technical reason could be found, these outliers were retained for the further statistical analyses.

Quantification of between-unit heterogeneity was accomplished by the 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 that mean values per bottle follow at least a unimodal distribution and results for each bottle that follow unimodal distribution with approximately the same standard deviation. The distribution of the mean values per bottle was visually evaluated using histograms and normal probability plots. Too few data are available for the bottle means to make a clear statement of the distribution. Therefore, it was visually checked

whether the 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 inhomogeneity that could be hidden by method intermediate precision, was calculated as described by Linsinger *et al.* [14]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

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

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

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

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

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

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

Table 3: Results of the homogeneity studies

CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF434c	16.7	2.2	5.2
ERM-BF434d	7.1	4.3	2.1
ERM-BF434e	6.0	2.7	2.6

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

4.2 Within-unit homogeneity and minimum sample intake

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

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

ERM-BF434a and ERM-BF434b are pure non-GM and GM materials. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. Based on the PCR measurements carried out on these two powders, it was concluded that 200 mg are a suitable minimum sample intake for PCR-based measurements.

5 Stability

Time, temperature and light were regarded as the most relevant influences on stability of the materials. The influence of ultraviolet or visible light was minimised by the choice of the containment which reduces light exposure. In addition, materials are stored and dispatched in the dark, thus eliminating practically the possibility of degradation by light. 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 under these conditions must be demonstrated, if transport without cooling will be applied.

The ERM-BF434e material was selected for the short-term stability study because it is a mixture of both GM and non-GM starting materials and makes it possible to assess the stability of each of them. Moreover, it is the mixture with the highest GM mass fraction, enabling the best method intermediate precision ($s_{wb,rel}$) of all three mixtures (Table 3).The short-term stability study was carried out using an isochronous design [15]. In this approach, samples of ERM-BF434e were stored for a certain time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under intermediate precision conditions.

The long-term stability study and estimation of the uncertainty was basis of a 6 months stability study which will be prolonged over a period of one year.

5.1 Short-term stability study

For the short-term stability study, units of ERM-BF434e were stored at -20 °C, 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 extraction replicates were measured by real-time PCR. The measurements were performed under intermediate precision conditions with respect to the PCR plates, and in a randomised sequence 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 on a confidence level of 99 %.

No statistical outliers were detected for the analyte, and the results were retained for the estimation of u_{sts}.

Furthermore, the data were evaluated against storage time and regression lines of mass fraction versus time were calculated, to test for potential increase/decrease of the 73496 rapeseed 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 99 % confidence level for any of the temperatures.

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

The results of the measurements are shown in Annex B.

5.2 Long-term stability study

Data from the stability study for GMO CRMs were obtained from the 6 months stability study of the ERM-BF434e. The units were stored at -70 °C, -20 °C and 4 °C. Three studies were performed for 0, 3 and 6 months (at each temperature). Five units per storage time and temperature were selected using a random stratified sampling scheme. Two extraction replicates per unit were randomized over three PCR plates under intermediate precision conditions. In fact, each of these studies can be seen as a two-point isochronous study. The evaluation is based on the ratio of samples from +4 °C and -70 °C.

The results were screened for outliers using the single and double Grubbs test. Two outliers were detected (-20 °C). As no technical reason could be found, these outliers were retained for the further statistical analyses. The long-term stability uncertainty contributions for -20 °C and 4 °C were estimated ($u_{lts,rel}$ for -20 °C, 6 months = 4.2 %; $u_{lts,rel}$ for 4 °C, 6 months = 5.2 %, see Table 4). Based on these data, it was concluded that the dried rapeseed 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 dispatch and 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 calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{tt}$$ **Equation 4**

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum (x_i - \bar{x})^2}} \cdot t_{sl}$$ **Equation 5**

- RSD relative standard deviation of all results of the stability study
- x_i result at time point *i*
- $\bar{x}$ mean results for all time points
- t_{tt} chosen transport time (1 week at 60 ºC)
- t_{sl} chosen shelf life (6 months at 4 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for 1 week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the stability study of ERM-BF434e rapeseed. The uncertainty contribution describes the possible degradation during 6 months storage at 4 °C.

The results of these evaluations are summarised in Table 4.

Table 4: 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 6 months

Candidate CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-BF434	1.1	5.2

The long-term stability study will continue over a period of one year, that might have an impact on the recent stability data and the $u_{lts,rel}$ value.

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

The material characterisation was based on a primary method of measurement confirmed by independent analysis. Gravimetric mixing was chosen as the method of choice. The five candidate CRMs under the label ERM-BF434 are rapeseed powder materials processed from non-GM and GM seeds of 73496 rapeseed. While ERM-BF434a is prepared from the pure blank material and ERM-BF434b from the pure GM material, the other candidate CRMs of the ERM-BF434 series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF434 is certified for the mass fraction of 73496 rapeseed.

6.1 Purity of the materials

The purity studies of the GM and non-GM batches were performed by Pioneer before the seeds were milled. These data of the purity were used for calculating the certified values of the produced reference materials. One seed out of 399 in the GM 73496 rapeseed material was detected as negative for the event 73496 (Section 3.1). No indication for the presence of 73496 was found in the non-GM material.

The same real-time PCR method was used by IRMM after in-house validation in order to further investigate the purity check of the non-GM seed starting powder. Real-time PCR measurements on the non-GM rapeseed powder were performed with a limit of detection (LOD) for the mass fraction of 0.04 g/kg. The method did not detect the event 73496 rapeseed (Section 3.5). The LOD of the method was used to establish the certified value of ERM-BF434a (Section 7).

As a consequence 100 % purity was used for the non-GM material. For the calculation of the certified mass fraction of 73496 in the powder mixtures 99.75 % purity was used for the GM material with 98.81 % representing the lower limit of 95 % confidence interval, Section 3.1).

6.2 Mass fractions and their uncertainties

The certified mass values are based on the mass fractions of dry-mixed GM and non-GM powders, corrected for their water mass fractions, and taking into account the powders' purity with regard to the 73496 event. The values were calculated according to the following equations:

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

$$m_{\text{GM,anhyd}}\,[\text{g}] = m_{\text{GM}}\,[\text{g}] \times (1 - \text{WMF}_{\text{GM}}\,[\text{g/g}]) \qquad \textbf{Equation 7}$$

$$m_{\text{nonGM,anhyd}}\,[\text{g}] = m_{\text{nonGM}}\,[\text{g}] \times (1 - \text{WMF}_{\text{nonGM}}\,[\text{g/g}]) \qquad \textbf{Equation 8}$$

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

In Table 5, the data supporting the calculation of the mass fractions of 73496 rapeseed are summarised.

Table 5: Subsequent mixing of GM powder with non-GM powder to prepare ERM-BF434c, d and e

CRM	GM powder			Non-GM powder		Mixtures
	Mass fraction [1] [g/kg]	Water mass fraction ± *U* (*k* = 2) [g/kg]	Mass [g]	Mass [2] [g]	Water mass fraction ± *U* (*k* = 2) [g/kg]	Calculated mass fraction [g/kg]
ERM-BF434e	1000.0 [3]	41.8 ± 1.2	363.4	3236.4	31.0 ± 0.9	99.69
ERM-BF434d	100.0 [4]	32.3 ± 0.9	360.6	3239.2	29.7 ± 0.8	9.96
ERM-BF434c	10.0 [5]	30.4 ± 0.9	359.8	3239.8	30.0 ± 0.8	1.0

1) 99.75 % purity was used for the GM material to calculate certified mass fraction of 73496 in the powder mixtures.

2) 100% purity was used for the non-GM powder used for the gravimetric preparations.

3) For the preparation of ERM-BF434e the 1000 g/kg GM powder was used.

4) For the preparation of ERM-BF434d the 100 g/kg GM powder (ERM-BF434e) was used.

5) For the preparation of ERM-BF434c the 10 g/kg GM powder (ERM-BF434d) was used.

The uncertainties on the certified mass fractions *(u_{char})* of 73496 rapeseed are composed of several contributions, i.e. the uncertainty on the mass determination ($u_{char,1}$), the uncertainty on the water mass fraction analysis ($u_{char,2}$), and the uncertainties on the purity determination of the non-GM and GM starting materials ($u_{char,3}$ and $u_{char,4}$). Based on a statistical analysis of the probability distribution to find a negative seed in the GM base material, it could be concluded that the purity was higher than 98.81 % (95 % confidence level, Section 3.1). This value was taken into account when estimating the uncertainty of the certified value (Table 6).

Table 6: Uncertainty budgets for the mass fractions of 73496 rapeseed in ERM-BF434

CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]				Combined uncertainty u_{char} [g/kg]
		$u_{char,1}$ [1)]	$u_{char,2}$ [2)]	$u_{char,3}$ [3)]	$u_{char,4}$ [4)]	
ERM-BF434a	0	n.a.	n.a.	0.0115	n.a.	0.0115
ERM-BF434b	1000	n.a.	n.a.	n.a.	2.4275	2.4275
ERM-BF434c	1	0.0022	0.0012	0.0115	0.0024	0.0121
ERM-BF434d	10	0.0180	0.0101	0.0115	0.0242	0.0338
ERM-BF434e	100	0.1269	0.0825	0.0115	0.2420	0.2857

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

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

3) Standard uncertainty of the purity estimation of the non-GM starting 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 starting material (> 98.81 %), based on the half-width interval between 98.81 % and 99.75 % divided by the square root of 6 (triangular distribution).

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 candidate CRMs (Section 3.4).

7 Value Assignment

Certified values were assigned. Certified values are values that fulfil the highest standards of accuracy. Full uncertainty budgets in accordance with the “Guide to the Expression of Uncertainty in Measurement” [4] were established.

The certified values are based on the masses of dried 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 5).

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

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

- 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 5.3
- u_{lts} was estimated as described in Section 5.3

For the blank material, the LOD of the method was used to describe the 95 % confidence interval on the certified mass fraction of the event (< 0.04 g/kg). This is supported by the high purity of the non-GM material and the absence of any mixing step; calculating the U_{CRM} for the blank material on the basis of the only quantifiable standard uncertainty ($u_{\text{char,3}}$) would result in a value of U = 0.02 g/kg (assuming k = 2), which is below the certified < 0.04 g/kg value. The LOD is, therefore, a conservative estimate of the certified value and its uncertainty.

A coverage factor k of 2 was applied to obtain the expanded uncertainties. The certified values and their uncertainties are summarised in Table 7.

Table 7: Certified values and their uncertainties for ERM-BF434

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] 3)
BF434a	< 0.04 1)	0.0115	n.a.	n.a.	n.a.	-
BF434b	> 988 2)	2.4275	n.a.	n.a.	n.a.	-
BF434c	1.00	0.0121	0.0518	0.0109	0.0518	0.15
BF434d	10.0	0.0338	0.4284	0.1096	0.5180	1.4
BF434e	100	0.2857	2.6915	1.0966	5.1837	12

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

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

3) Expanded (k = 2) and rounded uncertainty.

The relation between the certified GM powder mass fractions and the corresponding DNA copy number ratio is not known. The user is reminded that IRMM only certifies these materials for their mass fraction of 73496 rapeseed. 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 rapeseed variety tested.

8 Metrological traceability and commutability

8.1 Metrological traceability

The traceability chain for ERM-BF434c, d and e, is based on the use of calibrated balances and a thorough control of the weighing procedure. The value is therefore traceable to the SI. The certified values for the pure GM and non-GM reference materials (ERM-BF434a and ERM-BF434b) were retrieved from the purity assessment using event-specific 73496 real-time PCR.

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 analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called “commutability of a reference material”. There are various definitions expressing this concept. For instance, the CLSI Guideline C-53A [17] recommends the use of the following definition for the term *commutability*:

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

The commutability of a CRM defines its fitness for use and, thus, is a crucial characteristic in case of the application of different measurement methods. When commutability of a CRM is not established, 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 a calibrant.

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

9 Instructions for use

9.1 Safety and protection of the environment

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

9.2 Storage conditions

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

9.3 Minimum sample intake

The minimum sample intake is 200 mg.

ERM-BF434a and ERM-BF434b are pure non-GM and GM materials. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity.

9.4 Use of the certified value

The main purpose of these materials is the use for calibration or quality control of 73496 rapeseed detection methods. As any reference material, they can also be used for control charts or validation studies.

The user is reminded that this reference material is certified for its 73496 rapeseed mass fraction and should be used for measurements expressed in mass fractions. The measurement unit should not be changed especially as a significant difference (95 % confidence level) between the DNA mass fractions in the two powders used for the production of the gravimetric mixtures in ERM-BF434c, d and e was found.

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-BF434 for calibration and for quality control.

Comparing an analytical result with the certified value

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

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

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

Use in quality control charts

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

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Annexes

Annex A: Results of the homogeneity measurements

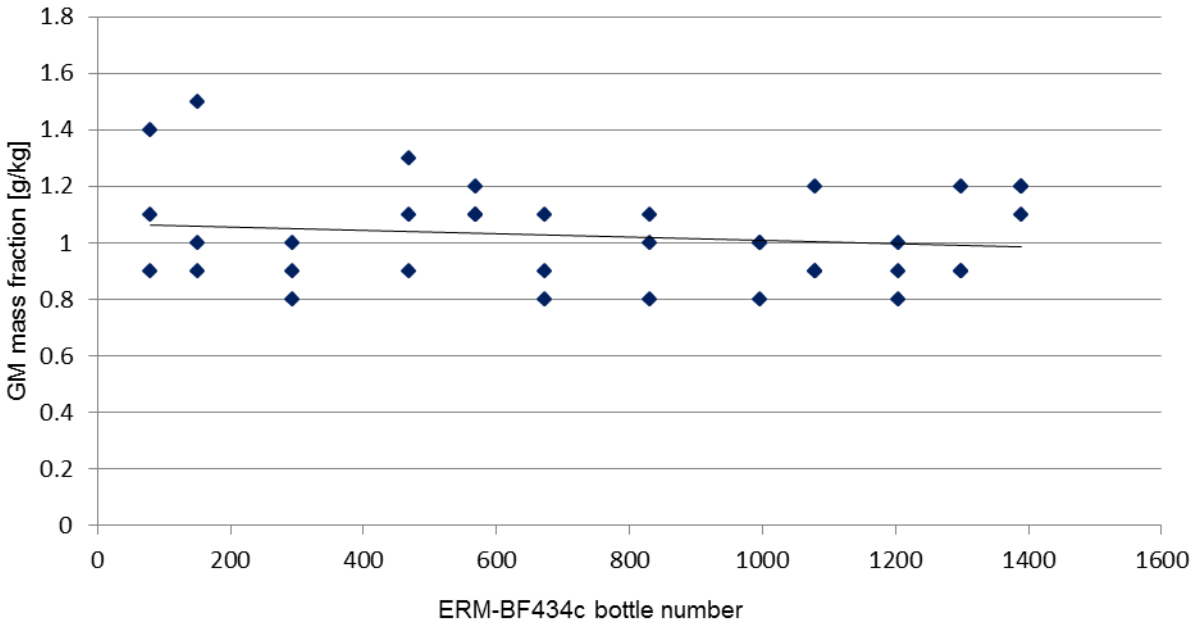

Figure A1: Real-time PCR measurement results obtained for ERM-BF434c (*N* = 12, *n* = 3, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

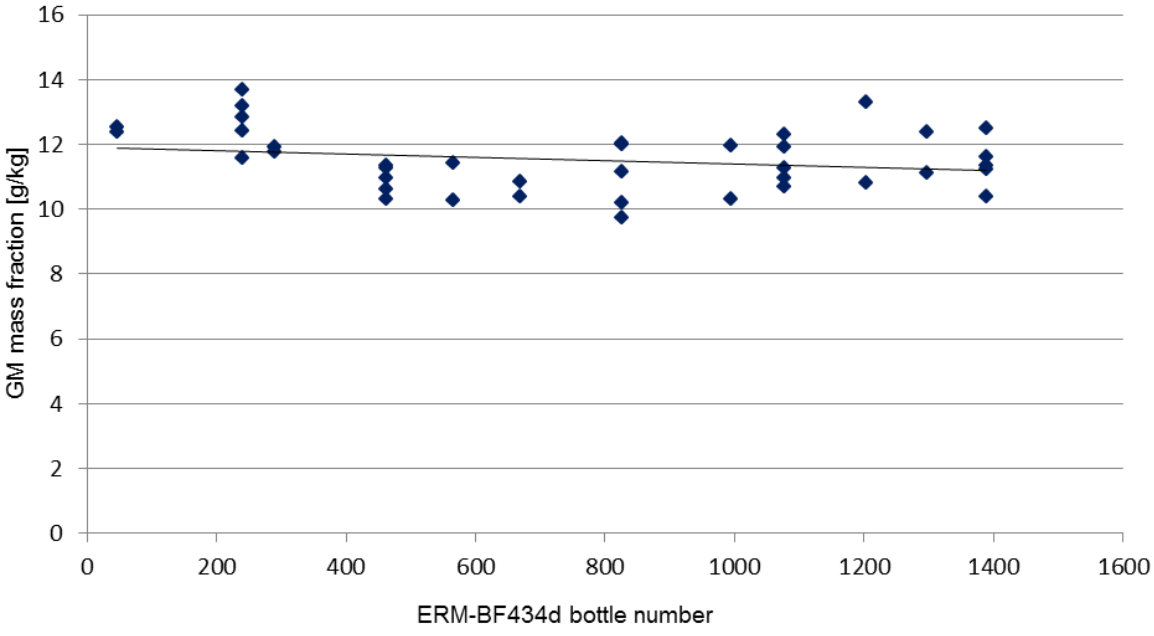

Figure A2: Real-time PCR measurement results obtained for ERM-BF434d (*N* = 12). Five of the samples were measured five times (*N* = 5, *n* = 5), while seven were measured twice (*N* = 7, *n* = 2). All measurements were carried out in triplicate on real-time PCR plate. The linear regression for all data points is given.

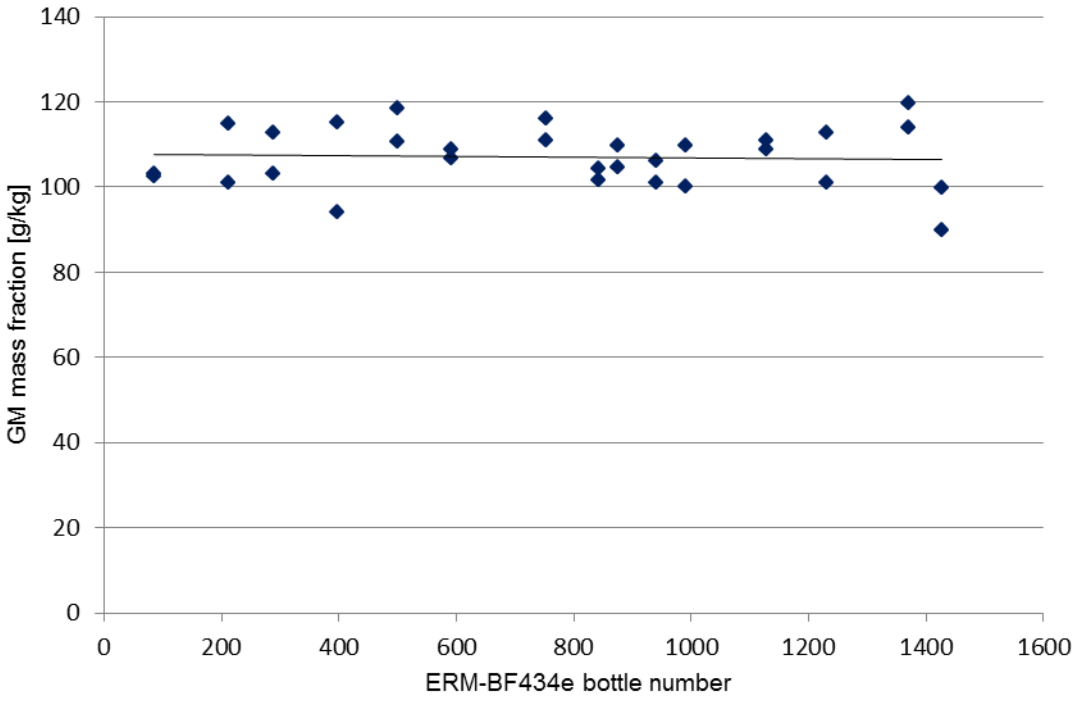

Figure A3: Real-time PCR measurement results obtained for ERM-BF434e (*N* = 15, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

Annex B: Results of the short-term stability measurements

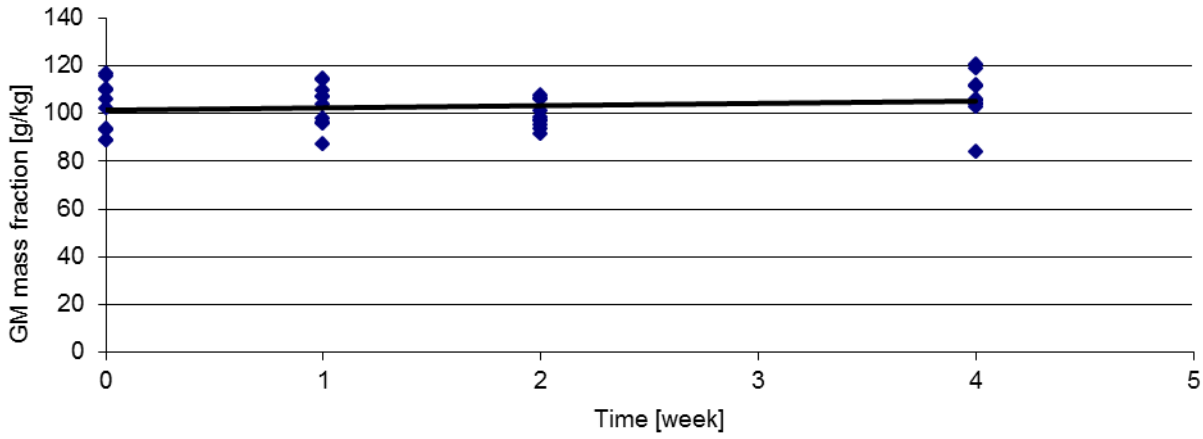

Figure B1: Real-time PCR measurement results obtained for ERM-BF434e (100 g/kg) during short-term stability testing at -20 °C (*N* = 5, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

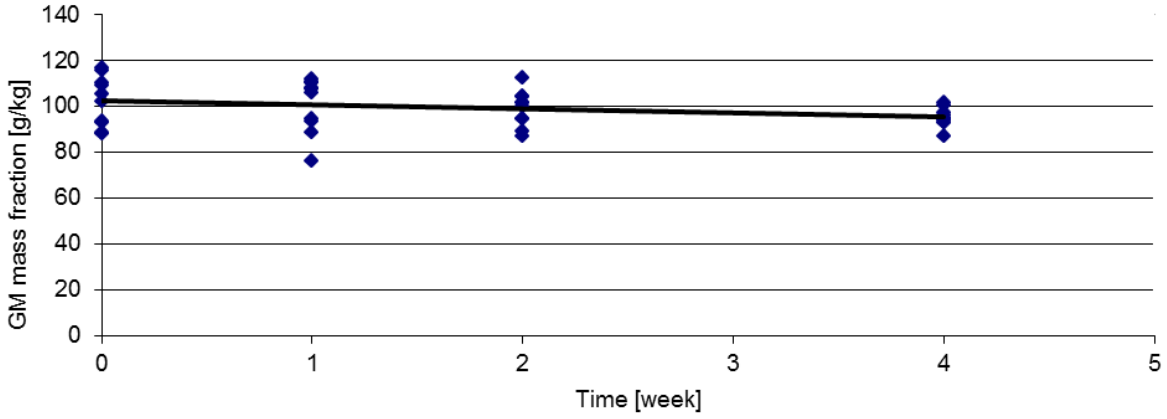

Figure B2: Real-time PCR measurement results obtained for ERM-BF434e (100 g/kg) during short-term stability testing at 4 °C (*N* = 5, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

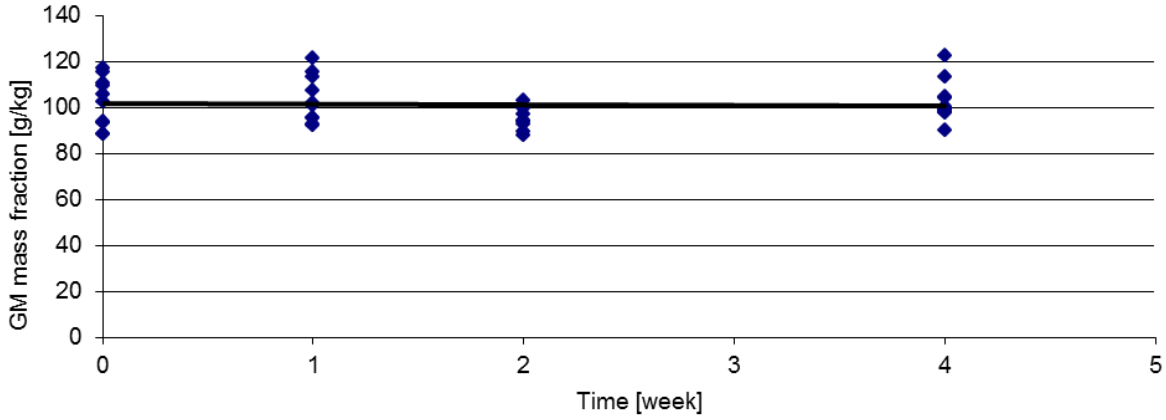

Figure B3: Real-time PCR measurement results obtained for ERM-BF434e (100 g/kg) during short-term stability testing at 18 °C (*N* = 5, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

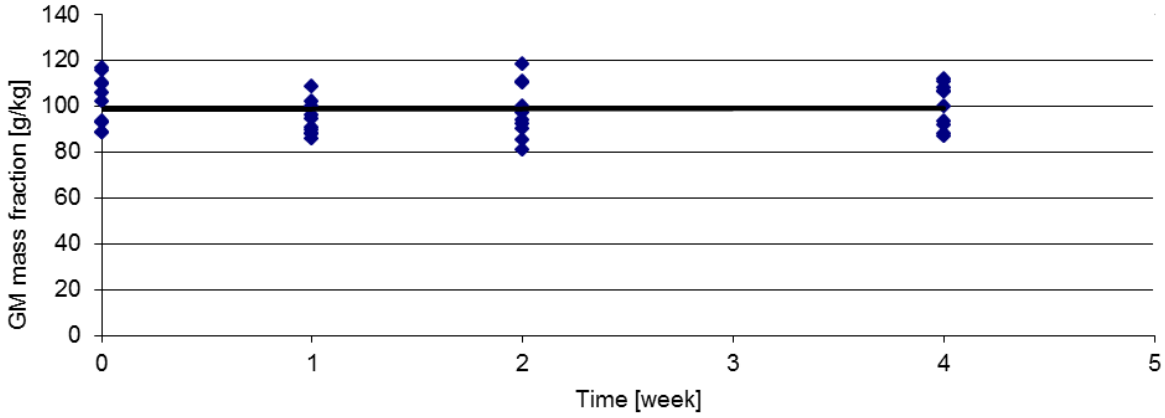

Figure B4: Real-time PCR measurement results obtained for ERM-BF434e (100 g/kg) during short-term stability testing at 60 °C (*N* = 5, *n* = 2, measured in triplicate on real-time PCR plate). The linear regression for all data points is given.

Annex C: Results of the long-term stability measurements

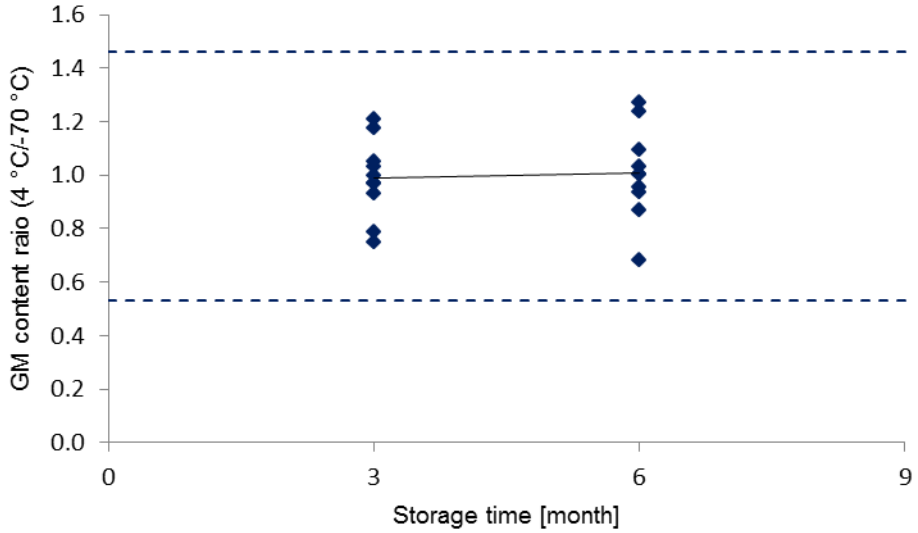

Figure C1: Real-time PCR measurement results obtained for ERM-BF434e (100 g/kg) during the long term stability study. The dashed lines give the limits of 3*s* obtained for the measurement results. The linear regression for all data points is given.

European Commission

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Title: The certification of different mass fractions of the GM event 73496 in rapeseed powder Certified Reference Materials ERM®-BF434a, ERM®-BF434b, ERM®-BF434c,ERM®-BF434d and ERM®-BF434e

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Abstract
This report describes the production of the set of Certified Reference Materials (CRMs) ERM-BF434a, b, c, d and e. These are matrix materials certified for their 73496 rapeseed mass fractions. Materials have been produced following ISO Guide 34:2009.
Genetically modified (GM) rapeseeds of the event 73496 and of a non-GM rapeseed variety were ground to obtain GM and non-GM starting materials. Gravimetric mixtures of non-GM and GM rapeseed powder were prepared by dry-mixing.
The between unit-heterogeneity has been quantified and stability during dispatch (short-term stability) and stability during storage (long-term stability) have been assessed in accordance with ISO Guide 35:2006.
The certified value was obtained from the gravimetric preparations, taking into account the purity of the seeds used for the processing of the materials and their respective water mass fraction. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements within the scope of accreditation to ISO/IEC 17025:2005).
Uncertainties of the certified values were calculated in compliance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties related to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).
The materials are intended for the calibration or quality control of 73496 rapeseed identification and quantification measurements expressed in mass fractions. 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 rapeseed powder closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.
The CRM has been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

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