Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

The Certification of Different Mass Fractions of DAS-68416-4 in Soya Seed Powder

Certified Reference Materials ERM®-BF432 (ERM®-BF432a, ERM®-BF432b, ERM®-BF432c, ERM®-BF432d)

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The Certification of Different Mass Fractions of DAS-68416-4 in Soya Seed Powder

Certified Reference Materials ERM®-BF432 (ERM®-BF432a, ERM®-BF432b, ERM®-BF432c, ERM®-BF432d)

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Summary

This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF432a, b, c and d, matrix materials certified for their DAS-68416-4 mass fractions. The material has been produced following ISO Guide 34:2009 [1].

Genetically modified (GM) seeds of the soya event DAS-68416-4 and of a non-GM soya variety were ground to obtain GM and non-GM base powders. Gravimetric mixtures of non-GM and GM soya 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 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 heterogeneity, instability, and characterisation.

The materials are intended for the calibration or quality control of 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 soya seed powder and closed under argon atmosphere. The minimum amount of sample to be used is 500 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:

	DAS-68416-4 Mass Fraction [1]	
	Certified value [2] [g/kg]	Uncertainty [3] [g/kg]
ERM-BF432a	< 0.3	-
ERM-BF432b	5.0	0.6
ERM-BF432c	10.0	1.7
ERM-BF432d	100	13

1) Genetically modified soya event with the unique identifier DAS-68416-4.

2) Mass fraction of DAS-68416-4 soya based on the masses of genetically modified DAS-68416-4 soya seed powder and non-modified soya seed 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.

Table of content

Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression y = a + bx
CRM	Certified reference material
DNA	Deoxyribonucleic acid
EC	European Commission
ERM®	Trademark of European Reference Materials
EU	European Union
EURL-GMFF	European Union Reference Laboratory for Genetically Modified Food and Feed
GM	Genetically modified
GMO	Genetically modified organism
GUM	Guide to the Expression of Uncertainty in Measurements *[ISO/IEC Guide 98-3:2008]*
IRMM	Institute for Reference Materials and Measurements of the JRC
IEC	International Electrotechnical Commission
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
t	Time
t_i	Time point for each replicate
TG	Thermogravimetry
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
u	standard uncertainty
U	expanded uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-unit heterogeneity that could be hidden by method repeatability; 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 demands the labelling of food and feed products consisting of or containing "more than 0.9 % genetically modified organisms" (GMOs) [5]. This is the labelling threshold level for food and feed consisting of GMOs that are being placed on the market in the European Community in accordance with the Community procedure. 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.

Dow AgroSciences (Oxon, UK) has developed the genetically modified (GM) soya event DAS-68416-4 (unique identifier code following Commission Regulation (EC) No 65/2004 [6]) and has asked in 2010 the Institute for Reference Materials and Measurements (IRMM, Geel, BE) to produce a reference material for the quantification of DAS-68416-4 soya. The event DAS-68416-4 expresses the AAD-12 and PAT proteins in soya, derived from *Delftia acidovorans* and *Streptomyces viridochromogenes*, providing tolerance to application of aryloxyalkanoate herbicides (such as 2,4-D) and glufosinate-ammonium, respectively [7]. The Certified Reference Material (CRM) produced by IRMM has been named ERM-BF432 and is composed of a set of four CRMs with different mass fractions of DAS-68416-4 soya.

1.2 Choice of the material

The set of CRMs ERM-BF432 was produced from ground GM seeds and non-GM seeds. Seeds were selected as source for the raw material due to their high purity, compared to harvest materials.

1.3 Design of the project

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

The different mass fractions of ERM-BF432 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 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, Dow AgroSciences supplied non-GM soya seeds and DAS-68416-4 soya seeds to IRMM. After arrival, the soya seeds were stored at (4 ± 3) °C in the dark until used for processing.

The purities of the delivered non-GM and GM soya seeds were investigated at IRMM using different approaches. For the GM soya seeds, a number of randomly selected seeds were analysed individually by real-time PCR to confirm the presence of the DAS-68416-4 event. Statistics was applied to calculate the purity of the GM seed batch. The purity of the non-GM soya seeds has been investigated after milling of the seeds on the powder material.

The purity of the delivered GM soya seed batch had been measured by Dow AgroSciences testing 300 individual seeds with a lateral flow test strip. All seeds tested positive. The purity was verified at IRMM by analysing 200 randomly selected GM seeds for the presence of the GM event DAS-68416-4. In order to avoid influences from adhering dust particles on the analytical results, seedlings were grown and genomic DNA was extracted from the leaves using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). Quantitative real-time PCR was then performed according to the event-specific real-time PCR method delivered under confidentiality agreement to IRMM. This method will be published after completion of its international validation on the homepage of the European Reference Laboratory for GM food and feed [8]. Genomic DNA extracted from pure DAS-68416-4 soya powder was used as positive control. Detection was done on an ABI 7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [9]. The results showed that all plants from the GM seed batch gave a signal for presence of the DAS-68416-4 event. Statistical analysis (Poisson distribution for rare events) revealed that the GM soya seed batch had a genetic purity of > 98.5 % (95 % level of confidence). The calculated lot purity of the GM seed batch was taken into account for the estimation of the uncertainties on the certified values of the reference materials (Section 6.2).

The purity of the non-GM seed batch was investigated on the processed powder. Real-time PCR measurements on the non-GM soya seed powder were performed with a limit of detection (LOD) for the mass fraction of 0.3 g/kg. The method did not detect the event DAS-68416-4 (Section 3.5). The LOD of the method was taken into account for the certified value (Section 6.1).

3.2 Processing

All soya seeds received by IRMM were rinsed with water, drained, and dried on special trays in a drying chamber of a freeze drier at 30 °C (Epsilon 2-65D, Osterode, DE). The drying time was about 20 h until the mass of the seeds before washing was diminished by about 3 %. The mass fraction of water was determined by volumetric Karl Fischer titration (V-KFT). After the washing and drying step, the non-GM seeds had a remaining residual water mass fraction of about 30 g/kg and the GM seeds had a remaining residual water mass fraction of about 16 g/kg.

About 25 kg of non-GM soya seeds and 8 kg of DAS-68416-4 soya seeds were finally used for the processing of ERM-BF432. The GM and non-GM base materials were processed separately. 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.

The seeds were milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). Prior to this milling step the soya seeds were frozen overnight in approximately 4 kg portions in stainless steal containers immersed in liquid nitrogen. The mill was also cooled down to process the seeds 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 1000 µm stainless steel mesh on a Russel Finex (London, UK). In case of the GM powder a coarse fraction of 11 g did not pass the 1000 µm mesh and was discarded. For the non-GM powder a coarse fraction of 35 g did not pass the 1000 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 soya tissues. The milling and sieving processes applied foster the separation of the different tissues from each other.

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 102 µm (s = 8 µm) for the GM powder and 120 µm (s = 7 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. For the non-GM and GM powders a residual water mass fraction of respectively (47.7 $\pm$ 6.8) g/kg and (57.3 $\pm$ 8.1) g/kg was measured by V-KFT with the expanded uncertainty calculated with k = 2. In order to reduce the water content, the powders were dried over night under vacuum in a freeze drier (Epsilon 2-65D, Osterode, DE) at 30 °C. The water mass fraction was measured once more and found low enough for dry-mixing. The final water mass fractions of the non-GM powder and the GM powder were (9.2 $\pm$ 1.3) g/kg (U, k = 2) and (8.2 $\pm$ 1.2) g/kg (U, k = 2) respectively (Table 5).

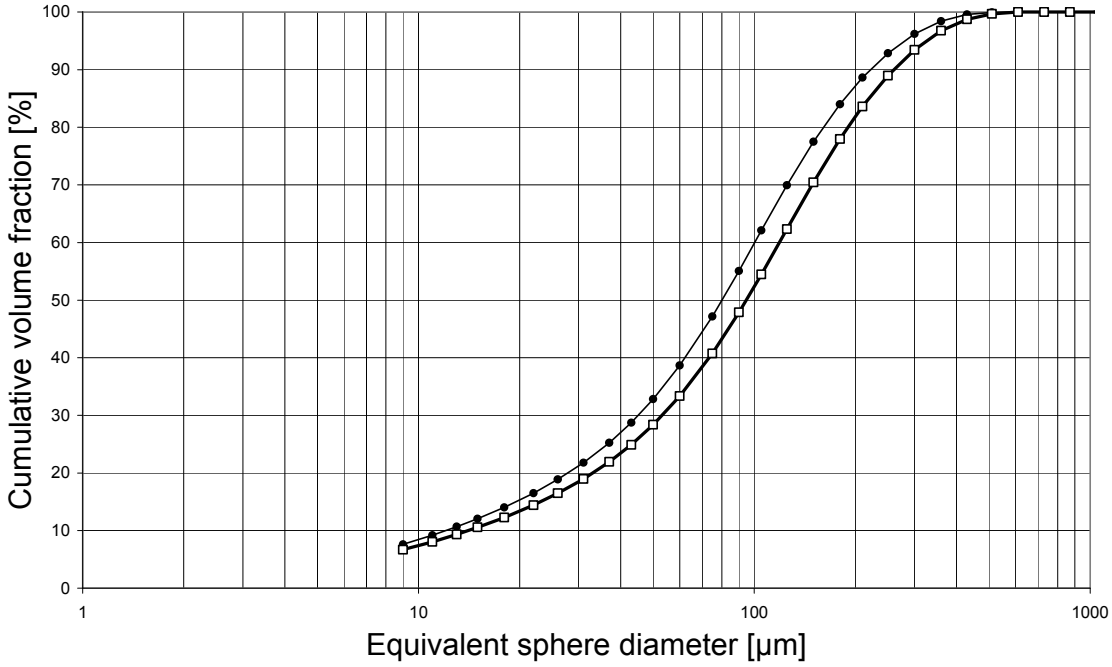

Figure 1: Accumulation of particle volume fractions in the GM powder (•) and non-GM powder (□) analysed by PSA. Each point represents the mean of five replicates (*N* = 1, *n* = 5). The total volume is set as 100 %.

The ground base materials were used to produce a blank material for DAS-68416-4 (non-GMO seed powder) and three mixtures containing different mass fractions of DAS-68416-4 soya seed powder in non-GM soya seed powder at nominal mass fraction levels of 5, 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, 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 by an external company accredited for ISO/IEC 17025 calibration services on an annual basis; additionally the performance of the balance is verified before daily use. Weighed portions of the powder materials were placed in one container, then turbula-mixed for 1 h, and further homogenised in a propeller mixer for an additional 2 min. The blank material was 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 DAS-68416-4/kg was produced by mixing pure GM with pure non-GM ground base materials. The material having a nominal mass fraction of 10 g DAS-68416-4/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder and the material with a nominal mass fraction of 5 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). The gravimetric preparation formed the basis for the calculation of the mass fraction of the powders (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 DAS-68416-4: nominal 0 g/kg = silver (BF432a), nominal 5 g/kg = blue (BF432b), nominal 10 g/kg = red (BF432c), nominal 100 g/kg = brown (BF432d), 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 ten randomly selected bottles from each of the powder materials was determined by volumetric Karl Fischer titration (V-KFT). The results are summarised in Table 1. As a result of the hygroscopic nature of the powders, it is recommended to close the vials immediately after taking a sample.

Table 1: Water mass fraction in candidate CRMs ERM-BF432 determined by volumetric KFT (*N* = 10, *n* = 1)

Candidate CRM	Water mass fraction [g/kg]	
	$x;^{-}$	*U (k = 2)*
ERM-BF432a	8.5	1.2
ERM-BF432b	8.7	1.2
ERM-BF432c	8.9	1.2
ERM-BF432d	8.7	1.2

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 870 µm (Figure 2). The mean particle diameters (*N* = 5, *n* = 2), calculated by the PSA software, were 117 µm (*s* = 12 µm), 128 µm (*s* = 8 µm), 101 µm (*s* = 18 µm) and 101 µm (*s* = 11 µm) for ERM-BF432a, b, c and d, respectively.

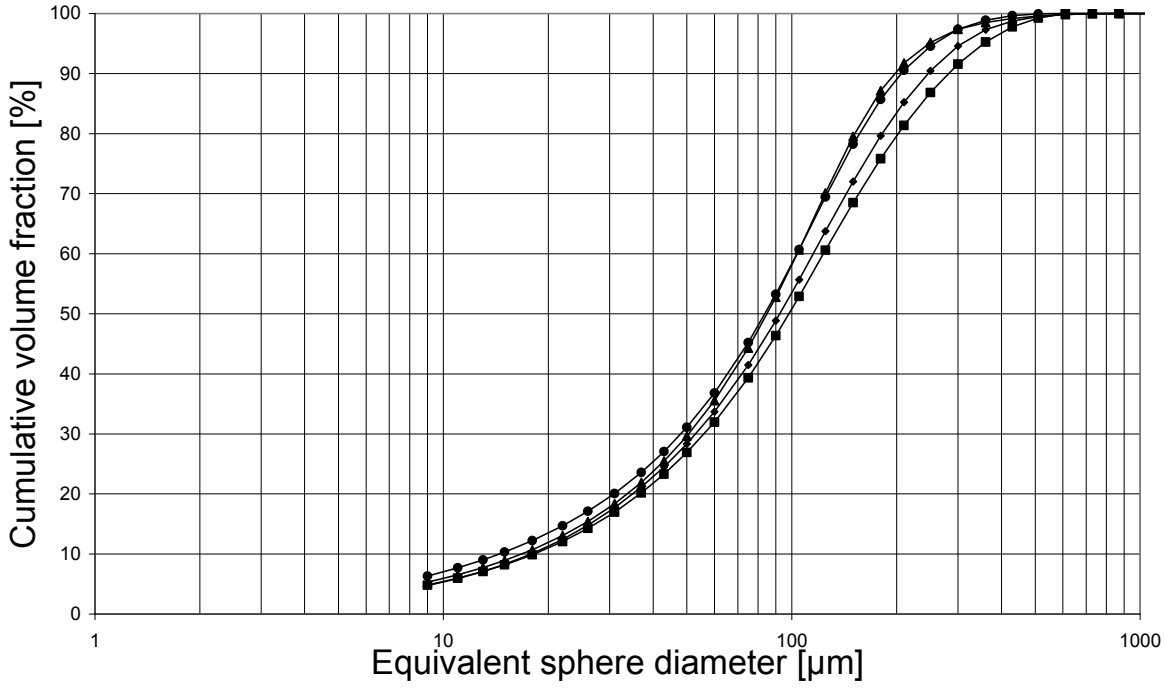

Figure 2: Accumulation of particle volume fractions in ERM-BF432a (◆), ERM-BF432b (■), ERM-BF432c (▲) and ERM-BF432d (●) analysed by PSA (*N* = 5, *n* = 2). Each point represents the mean of two replicate measurements (*N* = 1, *n* = 2). The total volume is set as 100 %.

3.4 DNA content of the base materials

Three of the described CRMs are mixtures of GM and non-GM soya seed 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 by 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 measured spectrophotometrically after derivatisation with diphenylamine. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [10, 11]. The extractable DNA mass fraction of the two materials was calculated as:

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

The ratio of the DNA mass extractable from 100 mg of GM and non-GM soya powder was found to be (1.16 ± 0.03), (*N* = 9 with an expanded uncertainty, *k* = 2). A *t*-test demonstrated that a significant difference between the DNA mass extracted from the GM and non-GM soya powders by the modified Ogur and Rosen method exists at 95 % confidence level. There is evidence that the extractable DNA mass in the two powders differ. Consequently the GM mass fractions of ERM-BF432 prepared by gravimetry are not equivalent to the GM DNA copy number measured by real-time PCR.

The DNA integrity was checked by gel electrophoresis. From 500 mg samples of the processed powder materials ERM-BF432a, BF432b and BF432c, DNA was extracted using a CTAB/Genomic DNA Tip 20 (Qiagen, Venlo, NL) DNA extraction method. Approximately 0.7 µg DNA were made visible on an agarose gel by UV. Staining was done with an ethidium bromide solution with a mass concentration of 7.5 g/L. A 1 kb DNA ladder (Invitrogen, Life Technologies Europe, Gent, BE) was used to estimate the DNA size. 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 DAS-68416-4 soya in all four CRMs was verified by the confidential real-time PCR method provided by Dow AgroSciences targeting the transgenic DNA insertion in this soya and using a sample intake of 1 g. At IRMM genomic DNA was extracted from 500 mg powder samples using a CTAB/Genomic DNA Tip 20 DNA extraction method. The real-time PCR test was calibrated with genomic DNA extracted from the pure DAS-68416-4 soya powder. After extraction the genomic DNA was diluted in a TE buffer solution (pH 8.0, 1 mmol/L TRIS and 0.01 mmol/L EDTA) and used to produce a calibration curve between 4-times diluted and 1000-times diluted and between undiluted and 100-times diluted for the target taxon-specific gene and the transgenic gene, respectively. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of DAS-68416-4 and the obtained Ct-values. For all standard curves, the efficiency was within the limits of the real-time PCR control chart. The LOD was calculated using the calibration curve approach estimating *s* on the lowest dilution measured. The results of the quantification of DAS-68416-4 are shown in Table 2. Quantification of the mass fraction of DAS-68416-4 in the powders by real-time PCR confirmed the consistency of the gravimetrically prepared mass fractions in ERM-BF432. 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 DAS-68416-4 soya powder. Consequently the deviation from the nominal mass fraction values (5, 10 and 100 g/kg) refers to a different DNA concentration in the two raw materials (Section 3.4); to a different extractability pattern or analytical behaviour of the taxon-specific target extracted from the non-GMO material or matrix effects caused by the GMO or the non-GMO material. The deviation between the certified mass fractions and the mass fraction quantified by real time PCR can also be caused by the sum of those concomitant effects.

Table 2: Quantification of the DAS-68416-4 soya mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure DAS-68416-4 seed powder for calibration

Candidate CRM	DAS-68416-4 soya mass fraction [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF432a	< 0.3 [1) 2)]	-
ERM-BF432b	5.5 [3)]	0.4
ERM-BF432c	12.0 [3)]	1.0
ERM-BF432d	108.0 [1)]	10.2

[1)] Mean for 2 samples from each of 5 random selected bottles (*N* = 5, *n* = 2), with each sample measured in three replicates.

[2)] The obtained value is below the LOD determined during method validation (0.3 g/kg).

[3)] Mean for 2 samples from each of 12 random selected bottles (*N* = 12, *n* = 2), with each sample measured in three replicates.

4 Assessment of 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 certified uncertainty. 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.

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

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 blank 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 CRM are valid for all bottles of the material, within the stated uncertainty.

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-BF432b and c. 15 bottles were chosen for ERM-BF432d in order to be able to facilitate the short-term stability study and the homogeneity study within one set of analysis. A random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12 or 15 groups (with similar number of bottles) and one bottle was randomly selected from each group. From each bottle, 2 independent samples were taken and analysed by real-time PCR. The measurements were performed under repeatability conditions, while the number of PCR plates resembled

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 tables in Annex A.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. Trends in the analytical sequence were found for ERM-BF432b, c and d and corrected. Additionally for ERM-BF432d a filling trend has been detected (99 % confidence level). This filling trend was not corrected for as it was minor. The significant (99 % confidence level) trends in the analytical sequence are pointing to a drift of the signal in the analytical system. As the analytical sequence and the unit numbers were not correlated, correction for these trends can improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. Therefore, the significant trends in the analytical sequence were corrected for:

$$corrected\,result = measured\,result - b \cdot i$$

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 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 method repeatability 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. Minor deviations from unimodality of the individual values do not grossly affect the estimate of between-unit standard deviations.

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 method repeatability, was calculated as described by Linsinger *et al.* [12]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum heterogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), 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 method repeatability.

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

CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF432b	7.8	5.1	3.5
ERM-BF432c	17.0	n.c.[1)]	7.8
ERM-BF432d	13.5	n.c. [1)]	5.8

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

The homogeneity study showed no outlying unit means and major 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 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 were performed using a 500 mg sample intake. This sample intake gives acceptable repeatability, 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 against 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 [13]. In that approach, samples of ERM-BF432d 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 repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

ERM-BF432 is a dried soya powder which has been processed similarly to other GMO CRM soya powders. As the water content of these powders and their particle size are similar, stability data obtained in the frame of the stability monitoring of soya GMO CRMs were used for the estimation of the uncertainty instead of an individual long-term stability study.

5.1 Short-term stability study

For the short-term stability study, samples 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, 2 samples were measured by real-time PCR. The measurements were performed under intermediate precision conditions with respect to the 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. No outliers were detected.

Furthermore, the data were plotted against storage time and regression lines of mass fraction versus time were calculated. No outliers were observed. 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 0 (99 % confidence level) for 4°C, 18 °C and 60 °C.

The results of the measurements are shown in Annex B.

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

5.2 Long-term stability study

Data from the stability-monitoring program for GMO CRMs were available. Soya powder CRMs (ERM-BF410, ERM-BF410k, ERM-BF425 and ERM- BF426) have been measured at 16 occasions over a period of 8 years. At each occasion, measurements were performed on units stored at +4 °C and -70 °C to eliminate between-day variation of results. 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 short-term stability showed that no outlying results were found and that no significant trend has been detected (99 % confidence level).

The results of the measurements are shown in Annex C.

To verify that the data obtained from stability monitoring can be used to estimate the stability uncertainty contribution for ERM-BF432, an additional isochronous study was organised within the frame of the short-term stability assessment (see Section 5.1). The data of the 4°C short-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 soya material can be stored at 4 °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 repeatability, 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 [14]. For this approach, the uncertainty of the linear regression line with a slope of zero is calculated. The uncertainty contribution u_{lts} or u_{sts} is then calculated as the product of the chosen shelf life or chosen dispatch time and the uncertainty of the regression lines as

$$u_{sts\ or\ lts,\ rel} = \frac{RSD}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{chosen}$$

RSD	relative standard deviation of all results of the stability study
t_i	time point for each replicate
$\bar{t}$	mean for all time points
t_{chosen}	shelf life (2 years at 4 ºC) or dispatch time (1 week)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies carried out for a time of 4 weeks. The uncertainty describes the possible change during a dispatch at 60 °C lasting for one week.

- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated for a storage at 4 °C. The uncertainty contribution therefore describes the possible degradation during 24 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 2 years

CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-BF432	2.1	2.5

The uncertainty of stability during dispatch at 60°C (short-term stability) did not give a significant trend. The material can be transported at ambient conditions without special precautions and no uncertainty contribution for the short-term stability needs to be taken into consideration when calculating the combined uncertainty.

The uncertainty of the long-term stability has been considered in the combined uncertainty (Section 7.1). 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) [15]) 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-BF432 gravimetric mixing was chosen as the method of choice. The four candidate CRMs under the label code ERM-BF432 are soya powder materials processed from non-GM and GM seeds. While ERM-BF432a is prepared from the pure blank material, the other CRMs of the series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF432 is being certified for the mass fraction of DAS-68416-4 soya.

6.1 Purity of the base materials

The purity of the GM and non-GM batches used for the processing of these powders was investigated in order to be able to calculate the certified value. No indication was found that the GM DAS-68416-4 soya base material contained seeds being negative for the event DAS-68416-4 (Section 3.1). No indication for the presence of DAS-68416-4 was found in the non-GM powder by real-time PCR (Section 3.5). As no evidence for a contamination was found in both base materials, 100 % purity was used for the calculation of the certified mass fraction of DAS-68416-4 in the powder mixtures.

The powder used for the production of ERM-BF432 did not contain traces of the DAS-68416-4 above the LOD of the applied real-time PCR method (Section 3.5). The certified value for ERM-BF432a is therefore based on the LOD of the real-time PCR method applied, as determined during in-house method validation.

6.2 Mass fractions and their uncertainties

The 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 DAS-68416-4 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 5, the data supporting the calculation of the mass fractions of DAS-68416-4 soya are summarised.

Table 5: Subsequent mixing of GM DAS-68416-4 soya seed powder with non-GM powder (ERM-BF432a) to prepare the ERM-BF432b, c and d materials

CRM	GM powder			Non-GM powder [1)]	Mixtures
	Mass fraction of GM powder [g/kg]	Water mass fraction $\pm$ U (k = 2) [g/kg]	Mass [g]	Mass [g]	Resulting mass fraction of GM powder [g/kg]
ERM-BF432d	1000.0	8.2 $\pm$ 1.2	399.6	3600.4	100.0
ERM-BF432c	100.0 [2)]	9.0 $\pm$ 1.3	400.0	3600.2	10.0
ERM-BF432b	10.0 [3)]	8.2 $\pm$ 1.2	1998.8	2001.0	5.0

[1)] The non-GM powder (ERM-BF432a) used for the gravimetric preparations had a water mass fraction of 9.2 $\pm$ 1.3 g/kg (U, k = 2) and was considered to be free of DAS-68416-4 soya.

[2)] For the preparation of ERM-BF432c, the 100 g/kg GM powder (ERM-BF432d) was used.

[3)] For the preparation of ERM-BF432b, the 10 g/kg GM powder (ERM-BF432c) was used.

The uncertainties on the certified mass fractions (u_{char}) of DAS-68416-4 soya is 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 base powders ($u_{char,3}$ 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 98.5 % (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 DAS-68416-4 soya in ERM-BF432

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-BF432a	**0**	n.a.	n.a.	0.0866	n.a.	**0.0866**
ERM-BF432b	**5**	0.0067	0.0065	0.0866	0.0216	**0.0897**
ERM-BF432c	**10**	0.0123	0.0113	0.0866	0.0432	**0.0982**
ERM-BF432d	**100**	0.0872	0.0924	0.0866	0.4323	**0.4588**

[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 volumetric KFT.

[3)] Standard uncertainty of the purity estimation of the non-GM base material (LOD = 0.3 g/kg), based on the half-width of the interval between 0 and 0.3 g/kg, divided by the square root of 3 (rectangular distribution).

[4)] Standard uncertainty of the purity estimation of the GM base material (> 98.5 %), based on the interval between 98.5 % and 100 % divided by the square root of 3 (rectangular distribution).

6.3 Verification 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.

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] must be established.

7.1 Certified values and their uncertainties

The certified values are based on the masses of dried powders 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 (Section 3.2 and Table 5 in Section 6.2).

The assigned uncertainty consists of uncertainties related to characterisation, u_{char} (Section 6.2), potential between-unit heterogeneity, u_{bb} (Section 4.1) and potential degradation during

transport (u_{sts}) and long-term storage, u_{lts} (Section 5). These different contributions were combined to estimate the expanded, relative uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor k as

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

- u_{char} was estimated as described in Section 6.2
- u_{bb} was estimated as described in Section 4.1
- u_{sts} and u_{lts} was estimated as described in Section 5.3. u_{lts} was taken into consideration, whereas u_{sts} is covered by the final assigned values and their uncertainties.

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 (< 0.3 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 ($u_{char,3}$) resulted in a value of U = x g/kg, which is below the certified < 0.3 g/kg value. The LOD is, therefore, already a conservative estimate of the certified value and no uncertainty is assigned.

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-BF432

CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{lts} [g/kg]	U_{CRM} [g/kg] [2)]
BF432a	< 0.3 [1)]	0.0866	n.a	n.a	-
BF432b	5.0	0.0897	0.2550	0.1250	0.6
BF432c	10.0	0.0982	0.7800	0.2500	1.7
BF432d	100	0.4588	5.7995	2.4998	13

[1)] With a 95 % confidence the certified value is below this level.
[2)] 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 DAS-68416-4. Additionally, one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements on unknown samples as DNA- and/or protein-based quantification of GMOs may vary with the particular matrix and the species variety tested.

8 Metrological traceability and commutability

8.1 Metrological traceability

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 [16] 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 in such cases, 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 calibrator.

Since the CRM is prepared from non-GM and GM soya powder, the DNA extracted from the CRM and from test samples is genomic DNA targeted by the event-specific PCR method. Consequently, commutability problems can be excluded.

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 seeds.

9.3 Minimum sample intake

The minimum sample intake is 500 mg.

9.4 Use of the certified value

The main purpose of these materials is the use for calibration or quality control of DAS-68416-4 soya 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-BF432 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 [17].

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-bottles 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

Table A1: Real-time PCR measurement results obtained for ERM-BF432b (*N* = 12, *n* = 2, measured in duplicate)

	Replicate 1		Replicate 2	
Individual unit number (filling sequence)	Sequence number (analytical sequence)	DAS-68416-4 soya mass fraction [g/kg]	Sequence number (analytical sequence)	DAS-68416-4 soya mass fraction [g/kg]
53	21	5.3	1	6.9
129	15	4.2	7	5.2
344	16	6.1	2	6.8
393	18	5.2	8	6.4
518	19	4.7	12	6
738	5	6.4	9	5.1
772	22	4.4	13	6
883	23	4.7	10	5.9
1084	3	5.8	24	4.3
1208	6	6	20	5.5
1325	14	5.3	4	4.7
1410	17	5.4	11	5

Table A2: Real-time PCR measurement results obtained for ERM-BF432c (*N* = 12, *n* = 2, measured in duplicate)

	Replicate 1		Replicate 2	
Individual unit number (filling sequence)	Sequence number (analytical sequence)	DAS-68416-4 soya mass fraction [g/kg]	Sequence number (analytical sequence)	DAS-68416-4 soya mass fraction [g/kg]
53	15	9.6	16	13.2
129	11	13.5	21	13.2
344	17	8.7	3	18.9
393	7	10.6	4	18.7
518	1	12.6	18	9.6
738	12	11.5	13	10.2
772	2	10.2	8	12.1
883	22	8.7	14	11.1
1084	9	11.1	5	17.1

1208	24	11.2	10	13.8
1325	19	10.7	23	11.6
1410	20	8.6	6	12.4

Table A3: Real-time PCR measurement results obtained for ERM-BF432d (*N* = 15, *n* = 2, measured in duplicate)

	Replicate 1		Replicate 2	
Individual unit number (filling sequence)	Sequence number (analytical sequence)	DAS-68416-4 soya mass fraction [g/kg]	Sequence number (analytical sequence)	DAS-68416-4 soya mass fraction [g/kg]
47	16	98.8	12	94.0
83	25	93.9	19	101.7
112	21	117.9	22	96.6
335	23	98.8	13	110.4
384	6	128.1	20	112.3
415	3	152.3	27	88.6
632	4	147.0	14	93.7
662	7	130.1	1	116.8
708	10	140.2	28	90.8
949	17	100.4	18	126.9
962	26	99.1	8	123.3
999	29	107.1	11	143.0
1242	24	104.4	5	169.9
1278	5	154.3	2	95.7
1318	30	109.7	15	112.1

Annex B: Results of the short-term stability measurements

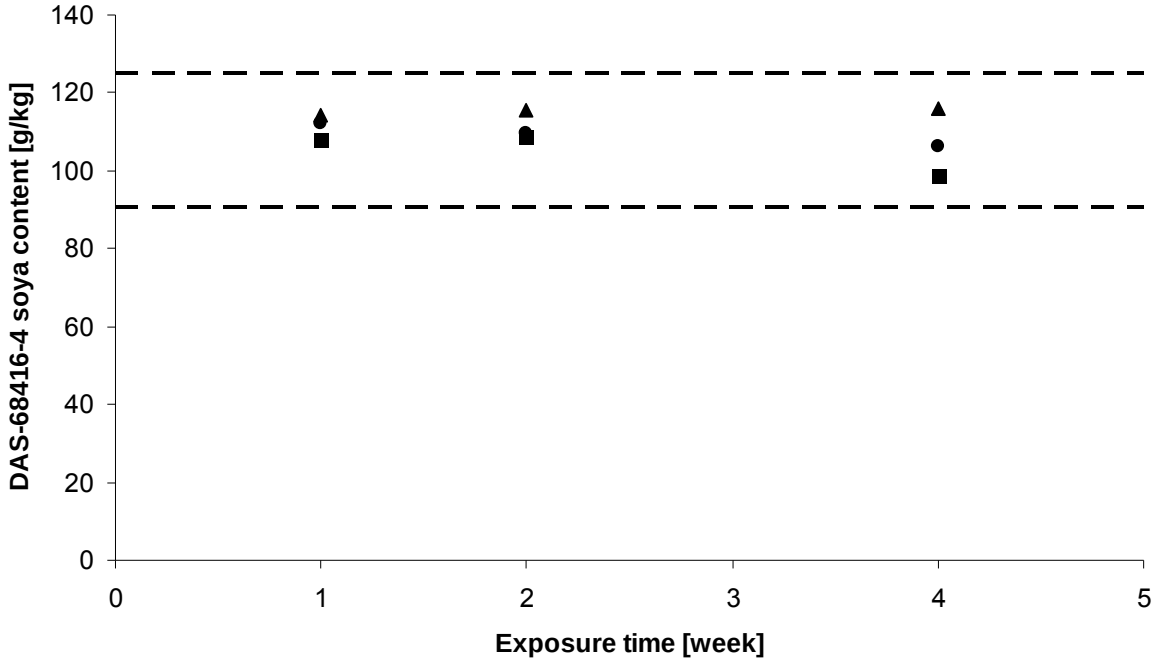

Figure B1: Real-time PCR measurement results obtained for ERM-BF432d during short-term stability testing (*N* = 5, *n* = 2) at 4°C (▲), 18°C (■) and 60°C (●). The dashed line gives the limits of 1 s obtained for the measurement results at the reference temperature of 70°C.

Annex C: Results of the long-term stability measurements

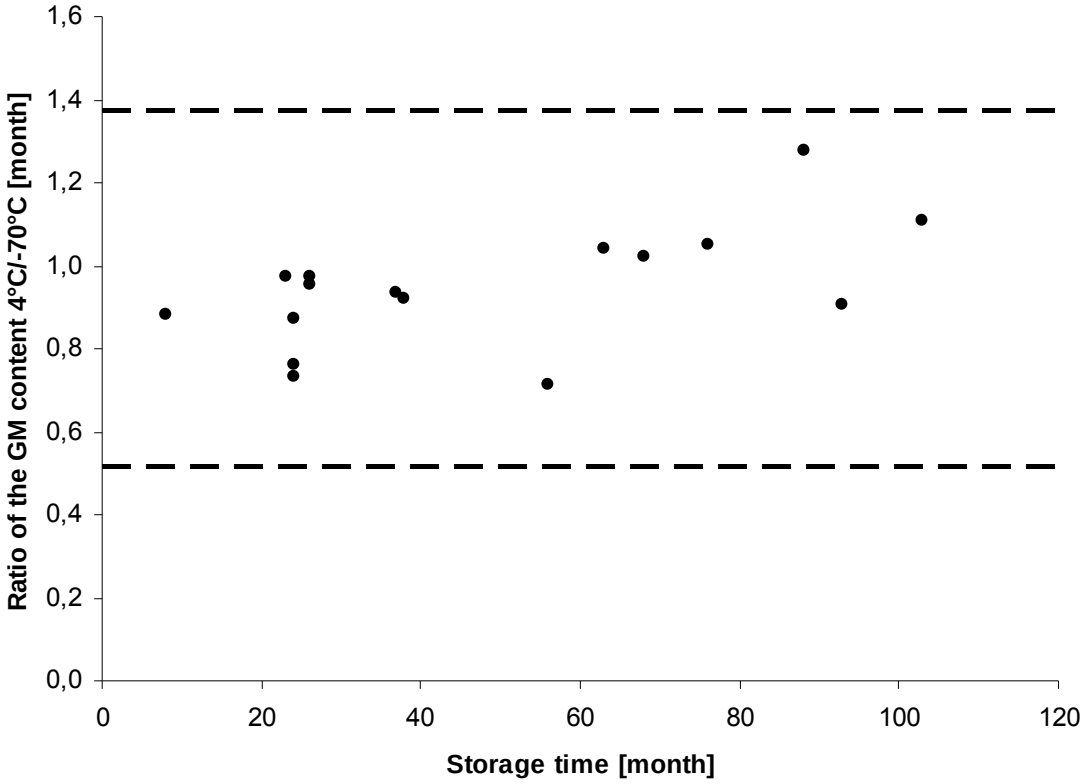

Figure C1: Real-time PCR measurement results obtained for ERM-BF410, BF410k, BF425 and BF426 during post certification monitoring. The dashed line gives the limits of 3 s obtained for the measurement results.

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Abstract

This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF432a, b, c and d, matrix materials certified for their DAS-68416-4 mass fractions. The material has been produced following ISO Guide 34:2009 [1].
Genetically modified (GM) seeds of the soya event DAS-68416-4 and of a non-GM soya variety were ground to obtain GM and non-GM base powders. Gravimetric mixtures of non-GM and GM soya 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 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 heterogeneity, instability, and characterisation.
The materials are intended for the calibration or quality control of 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 soya seed powder and closed under argon atmosphere. The minimum amount of sample to be used is 500 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:

	DAS-68416-4 Mass Fraction [1)]	
	Certified value [2)] [g/kg]	Uncertainty [3)] [g/kg]
ERM-BF432a	< 0.3	-
ERM-BF432b	5.0	0.6
ERM-BF432c	10.0	1.7
ERM-BF432d	100	13

1) Genetically modified soya event with the unique identifier DAS-68416-4.
2) Mass fraction of DAS-68416-4 soya based on the masses of genetically modified DAS-68416-4 soya seed powder and non-modified soya seed 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.

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