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CERTIFICATION REPORT

The certification of different mass fractions of DAS-81419-2 in soya seed powder

Certified Reference Materials ERM®-BF437a, ERM®-BF437b, ERM®-BF437c, ERM®-BF437d and ERM®-BF437e

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Summary

This report describes the production of a set of Certified Reference Materials (CRMs) ERM-BF437a, b, c, d and e, which are certified for their DAS-81419-2 mass fractions. This material was produced following ISO Guide 34:2009 [1] and is certified in accordance with ISO Guide 35:2006 [2].

Genetically modified (GM) seeds of the soya event DAS-81419-2 and seeds from a non-GM soya variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM soya powder were prepared gravimetrically.

The certified value was obtained from the gravimetric preparations, taking into account the genetic purity with respect to the event DAS-81419-2 of the two powder materials and their water mass fractions. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements were within the scope of accreditation to ISO/IEC 17025:2005 [3]).

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

The materials are intended for the calibration or quality control of real-time PCR measurements to identify DAS-81419-2 soya and/or quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs are available in glass vials containing at least 1 g of dried soya seed powder which were sealed under atmosphere of argon. The minimum amount of sample to be used is 200 mg.

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

The following values were assigned:

	DAS-81419-2 mass fraction 1)	
	Certified value [g/kg]	Uncertainty [g/kg] 5)
ERM-BF437a	< 0.07 2)	-
ERM-BF437b	> 986 3)	-
ERM-BF437c	0.99 4)	0.12
ERM-BF437d	9.9 4)	1.5
ERM-BF437e	100 4)	9

1) Genetically modified soya with the unique identifier DAS-81419-2.

2) The certified reference material has been produced from conventional, non-modified soya seeds. No contamination was detected in this material when using an event-specific real-time polymerase chain reaction targeting the DAS-81419-2 soya event. The limit of detection (LOD) was 0.07 g/kg. With 95 % confidence, the true DAS-81419-2 soya mass fraction of the material is below 0.07 g/kg. The certified value is traceable to the International System of units (SI).

3) The certified reference material has been produced from genetically modified DAS-81419-2 soya seeds. The certified value is based on the genetic purity of the soya powder with regard to DAS-81419-2 soya. In total 219 seeds were tested individually for the presence of the DAS-81419-2 soya event by event-specific real-time polymerase chain reaction. All seeds tested positive. With 95 % confidence, the true DAS-81419-2 soya mass fraction of the material is above 986 g/kg. The certified value is traceable to the International System of units (SI).

4) The certified value is based on the masses of dried genetically modified DAS-81419-2 soya powder and dried non-modified soya powder that were mixed, taking into account their respective genetic purity with regard to DAS-81419-2 soya and their respective water content. The certified value is traceable to the International System of units (SI).

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

Table of contents

Summary 1
Table of contents 3
Glossary 4
1 Introduction 7
1.1 Background: need for the CRM 7
1.2 Choice of the material 7
1.3 Design of the project 7
2 Participants 7
2.1 Provider of raw material 7
2.2 Project management, processing, analytical measurements and evaluation 8
3 Material processing and process control 8
3.1 Origin of the starting material 8
3.2 Processing and process control 8
3.3 DNA content of the base materials 12
3.4 Confirmation measurements 12
4 Homogeneity 13
4.1 Between-unit homogeneity 13
4.2 Within-unit homogeneity and minimum sample intake 15
5 Stability 15
5.1 Short-term stability study 16
5.2 Long-term stability study 16
5.3 Estimation of uncertainties 17
6 Characterisation 18
6.1 Genetic purity of the base materials 18
6.2 Mass fractions and their uncertainties 18
6.3 Verification measurements 20
7 Value Assignment 20
8 Metrological traceability and commutability 21
8.1 Metrological traceability 21
8.2 Commutability 21
9 Instructions for use 22
9.1 Safety and protection of the environment 22
9.2 Storage conditions 22
9.3 Minimum sample intake 22
9.4 Use of the certified value 22
Acknowledgments 24
References 25
Annexes 26

Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression y = a + *b*x
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
CRM	Certified reference material
Cry	Crystal toxin (protein)
CTAB	Cetyltrimethylammonium bromide
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]*
EDTA	Ethylenediamintetraacetic acid
IEC	International Electrotechnical Commission
IRMM	Institute for Reference Materials and Measurements
ISO	International Organization for Standardization
JRC	Joint Research Centre
k	Coverage factor
LOD	Limit of detection
MS_{between}	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit
N	Number of samples (units) analysed
n.a.	Not applicable
n.c.	Not calculated
PAT	Phosphinothricin-N-acetyltransferase
PCR	Polymerase chain reaction
PSA	Particle size analysis
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
rpm	Revolutions per minute
RT	Room temperature
s	Sample standard deviation
$s_{\bar{x}}$	Standard deviation of the estimate of the mean (also referred to as standard error of the estimate of the mean)
s_{bb}	Between-unit standard deviation; an additional index "rel" is added as appropriate
s_{rel}	Relative standard deviation (also referred to as RSD)
s_{wb}	Within-unit standard deviation; an additional index "rel" is added as appropriate
t	Time
t_i	Time point for each replicate
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation in real-time PCR
TE	Tris-EDTA
u	Standard uncertainty
U	Expanded uncertainty

u^{*}_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by the intermediate precision of the method; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
VIM	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
$\overline{y}$	Mean of all results of the homogeneity study
ν	Degrees of freedom

1 Introduction

1.1 Background: need for the CRM

The European Union has legislation which regulates placing on the market any food or feed which consists of, contains or is produced from genetically modified organisms (GMOs). These items are referred to as genetically modified food and feed and require authorisation for marketing in the European Union. They will additionally need to be labelled if they contain more than 0.9 % of GMOs [5]. This labelling threshold is applicable for the adventitious presence of GMOs, whilst GMOs that are specifically added need to be labelled independently from any threshold. However, feed may contain 0.1 mass percent of a GMO for which an authorisation process is pending, or for which authorisation in the EU has expired [6]. These thresholds require the development and validation of reliable methods for GMO quantification, and the production of reference materials for calibration or quality control of these methods.

Dow AgroSciences (Oxon, UK) has developed the genetically modified (GM) soya event DAS-81419-2 (unique identifier code following Commission Regulation (EC) No 65/2004 [7]). In 2013 they asked the Institute for Reference Materials and Measurements (IRMM, Geel, BE) to produce a certified reference material (CRM) for the quantification of DAS-81419-2 soya. The event DAS-81419-2 expresses the Cry1F, Cry1Ac and PAT proteins in soya, derived from *Bacillus thuringiensis* subsp. *aizawa*, *Bacillus thuringiensis* subsp. *kurstaki* and *Streptomyces viridichromogenes*, respectively. As a result, DAS-81419-2 soya is resistant against certain lepidopteran chewing insect pests and is tolerant to herbicides which contain glufosinate-ammonium [8]. The CRM produced by IRMM has been named ERM-BF437 and is composed of a set of five CRMs with different mass fractions of DAS-81419-2 soya.

1.2 Choice of the material

The set of CRMs ERM-BF437 was produced from milled GM seeds and non-GM seeds. Seeds were selected as the source for the raw material due to their high purity, as compared to harvest materials.

1.3 Design of the project

Alongside the pure non-GM material ERM-BF437a and the pure GM material ERM-BF437b, mixtures of non-GM and GM soya powder were prepared gravimetrically. The first mixed material ERM-BF437e was prepared by mixing pure GM with non-GM soya powder. ERM-BF437d was prepared by further dilution of ERM-BF437e, and ERM-BF437c was prepared by further dilution of ERM-BF437d, in both cases with non-GM soya powder.

The different mass fractions of ERM-BF437 were certified using a gravimetric approach, the details of which are described in Section 6.

2 Participants

2.1 Provider of raw material

Dow AgroSciences, Oxon, UK

2.2 Project management, processing, analytical measurements and evaluation

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM, and to ISO/IEC 17025 for GMO quantification, BELAC No. 268-TEST)

3 Material processing and process control

3.1 Origin of the starting material

Dow AgroSciences supplied the IRMM with non-GM soya seeds and DAS-81419-2 soya seeds to prepare candidate CRMs. According to the information provided by Dow AgroSciences, the DAS-81419-2 soya seeds are homozygous, following several generations of self-fertilisation. After arrival, the seeds were stored at (4 ± 3) °C in the dark until processing. The genetic purities with respect to the DAS-81419-2 event of the delivered non-GM and GM soya seeds were investigated at the IRMM as described below.

The genetic purity of the GM soya seeds was assessed by analysing 219 randomly selected seeds for the presence of the GM event DAS-81419-2. Genomic DNA was extracted from individual seeds using the DNeasy Plant Mini kit (Qiagen, Venlo, NL). To verify the presence of the DAS-81419-2 event in the seeds, an in-house validated qualitative real-time PCR method was then performed in accordance with the event-specific real-time PCR method delivered under a confidentiality agreement to the IRMM. This method will be published on the homepage of the European Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) once it has been internationally validated [9]. Genomic DNA extracted from pure DAS-81419-2 soya powder was used as positive control. Detection was performed on an ABI 7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [10]. The results showed that all the GM seeds tested gave a signal for the presence of the DAS-81419-2 event. Statistical analysis of the 219 measurements (Poisson distribution for rare events) revealed that the GM soya seed batch had a genetic purity of > 98.6 % (95 % level of confidence). This is in agreement with the 99.0 % genetic purity (95 % level of confidence) reported by Dow AgroSciences for the delivered GM seed material. The calculated genetic purity with respect to the DAS-81419-2 event of > 98.6 % was taken into account for the estimation of the uncertainties associated to the certified values of the CRMs (Section 6.2).

The genetic purity of the non-GM seed batch was investigated from the processed seed powder. Real-time PCR measurements on the non-GM soya seed powder were performed with a limit of detection (LOD) of 0.07 g/kg. The method did not detect the event DAS-81419-2 (Section 3.4). This is in agreement with the 99.9 % genetic purity (95 % level of confidence) reported by Dow AgroSciences for the delivered non-GM seed material. The LOD of the event-specific real-time PCR method established at IRMM was taken into account for calculating the certified value of ERM-BF437a (Section 7).

3.2 Processing and process control

All soya seeds received by the IRMM were rinsed with water, drained, and dried on trays in the drying chamber of a freeze-dryer at 30 °C for 20 h (Epsilon 2-100D, Osterode, DE). The mass fraction of water was determined by volumetric Karl Fischer titration (V-KFT) (758 KFD Titrino, Metrohm, Herisau, CH). After the washing and drying step, the non-GM seeds had a remaining residual water mass fraction of approximately 64 g/kg and the GM seeds had a remaining residual water mass fraction of approximately 61 g/kg.

Approximately 23 kg of non-GM soya seeds and 9 kg of DAS-81419-2 soya seeds were used for the production of ERM-BF437. The GM and non-GM base materials were processed separately. The risks of cross-contamination and contamination with foreign DNA were minimized by thorough cleaning, by wearing clean laboratory clothing and by using measures to prevent airborne cross-contamination. Additionally, all contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) prior to exposure to the materials. An in-house validation study had previously shown that the solution degraded DNA effectively under the conditions used. If required, the base powders were stored for short periods of time in closed plastic containers.

The soya seeds were frozen overnight in liquid nitrogen in approximately 6 kg portions in stainless steel containers and were subsequently milled using a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE). The mill was maintained below -90 °C throughout the process. The feeding speed of the mill was set to ensure that the milling was optimal with respect to the required particle size. After milling, the powder was maintained at -20 °C. The GM and non-GM powders were then sieved separately with a 750 µm stainless steel mesh on a sieving machine equipped with an ultra-sound sieving aid (Russel Finex, London, UK). A coarse fraction of 154 g and 215 g for the GM and non-GM powders, respectively, did not pass the 750 µm mesh and was discarded. The remaining powder from each base material was mixed in a DynaMIX CM200 (WAB, Basel, CH) for 1 h to homogenize the distribution of the different types of soya tissues, since the milling and sieving processes encourage the separation of the different tissues from each other.

For the non-GM and GM powders, residual water mass fractions (N = 1, n = 3) of (75.8 ± 4.9) g/kg and (90.1 ± 5.9) g/kg, respectively, were measured by volumetric Karl Fischer titration, with the expanded uncertainty calculated with a coverage factor of k = 2. To facilitate gravimetric mixing, the water content was further reduced. The powders were dried overnight under a vacuum in the freeze-dryer at 30 °C. The final water mass fractions (N = 1, n = 5) of the non-GM powder and the GM powder were (25.9 ± 1.6) g/kg (U, k = 2) and (25.6 ± 1.6) g/kg (U, k = 2), respectively. The particle volumes for both powders were measured based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE) and were compared (Figure 1). The cumulative volume distribution of the 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 will therefore overestimate the mean particle size. A three point specification of the particle size distribution (N = 1, n = 5) was calculated, consisting of the equivalent sphere diameters where 10 %, 50 % and 90 % of the volume distribution have a smaller particle size. These size classes are denoted X_{10}, X_{50} and X_{90}, respectively. For the non-GM powder, the values of X_{10}, X_{50} and X_{90} measured by laser diffraction were (15.1 ± 3.1) µm (U, k = 2), (89.3 ± 14.8) µm (U, k = 2) and (246.1 ± 50.2) µm (U, k = 2), respectively. For the GM powder, the values of X_{10}, X_{50} and X_{90} were (21.5 ± 4.4) µm (U, k = 2), (112.9 ± 18.7) µm (U, k = 2) and (286.7 ± 58.5) µm (U, k = 2), respectively. A t-test showed with 99 % confidence that no significant difference exists between the X_{10}, X_{50} and X_{90} values of the non-GM and GM soya powders. Therefore it was concluded that the particle volume distributions of the non-GM and GM base powders were sufficiently similar to allow the mixtures to be processed without introducing a bias related to the ability to extract DNA.

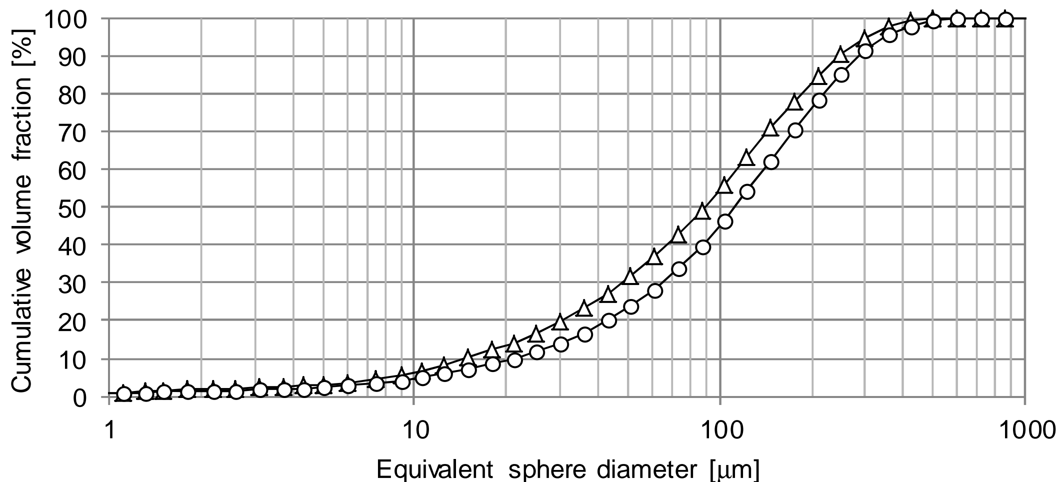

Figure 1: Cumulative representation of particle volume fractions in the GM powder (○) and non-GM powder (△) analysed by laser diffraction (N = 1, n = 5). The total volume for each material is set as 100 %.

The milled base materials were used to produce a blank material for DAS-81419-2 (non-GM soya seed powder), a pure GM DAS-81419-2 material and three mixtures at nominal mass fraction levels of 1, 10 and 100 g/kg DAS-81419-2 soya. The term "nominal" is used for the value targeted in the processing whereas the value assigned after completion of the certification process is called certified value.

All the materials produced were treated according to the same procedure. The powder materials were weighed using a calibrated balance with an intermediate precision, determined during calibration and expressed as standard uncertainty (u), of 0.2 g. 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 by DynaMIX CM200. The blank material was processed first, followed by the GM containing mixtures and the pure GM material. The masses of the non-GM and GM powders, which are theoretically needed to reach a certain nominal mass fraction, were calculated. They were also corrected for their respective water content. The material with a nominal DAS-81419-2 mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM milled base materials. The material with a nominal DAS-81419-2 mass fraction of 10 g/kg was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder and the material with a nominal mass fraction of 1 g/kg was 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 materials was taken into account (Table 5). During the certification process, the gravimetric preparation was the basis for the calculation of the certified DAS-81419-2 soya mass fraction for the three powder mixtures (Section 6).

Powders were put into 10 mL brown glass vials using an automatic filling device. The first 30 vials of each batch were discarded as an additional precaution against carry-over contamination. Lyophilisation inserts were automatically placed in the bottle necks. The vials were then placed in a freeze-dryer under an argon atmosphere. The vials were closed inside the freeze-dryer with the help of a hydraulic device and then sealed with aluminium caps to prevent them accidentally opening during storage and transport. Colour-coded caps were used to facilitate the identification of the different mass fraction levels of DAS-81419-2: nominal 0 g/kg = silver (BF437a), nominal 1000 g/kg = black (BF437b), nominal 1 g/kg = yellow (BF437c), nominal 10 g/kg = red (BF437d), nominal 100 g/kg = brown (BF437e), 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 stored in the dark at (4 ± 3) °C.

To determine the residual mass fraction of water in the candidate CRMs, five randomly selected bottles from each of the powder materials were measured by V-KFT. The results are summarised in Table 1.

Table 1: Water mass fraction in candidate CRMs ERM-BF437 determined by V-KFT ($N = 5$, $n = 2$). The associated expanded uncertainty (U) has been estimated during validation of the V-KFT method.

Candidate CRM	Water mass fraction [g/kg]	
	$\overline{x}$	U ($k = 2$)
ERM-BF437a	16.1	2.4
ERM-BF437b	22.6	3.4
ERM-BF437c	16.8	2.5
ERM-BF437d	16.2	2.4
ERM-BF437e	18.3	2.7

To determine the particle volume distribution in the candidate CRMs, five randomly selected bottles from each of the powder materials were analysed twice ($N = 5$, $n = 2$). The particle volume distribution was established based on their laser diffraction pattern. The powders have a particle diameter below 500 µm (Figure 2). The mean particle diameters and standard deviations of the mean, measured by laser diffraction, were 117 µm ($s_{\bar{x}}$ = 14 µm), 149 µm ($s_{\bar{x}}$ = 7 µm), 123 µm ($s_{\bar{x}}$ = 3 µm), 127 µm ($s_{\bar{x}}$ = 5 µm) and 136 µm ($s_{\bar{x}}$ = 13 µm) for ERM-BF437a, b, c, d and e, respectively.

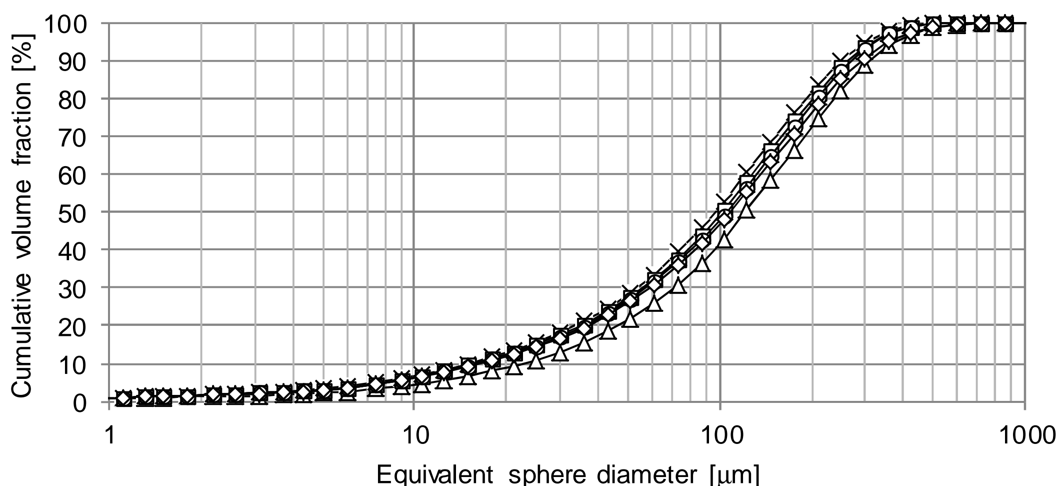

Figure 2: Cumulative representation of particle volume fractions in ERM-BF437a (x), ERM-BF437b (△), ERM-BF437c (□), ERM-BF437d (○) and ERM-BF437e (◇) analysed by laser diffraction ($N = 5$, $n = 2$). The total volume for each preparation is set as 100 %.

3.3 DNA content of the base materials

Three of the candidate CRMs are mixtures of GM and non-GM soya seed powders. Consequently, any difference in the extractable DNA mass fraction in the non-GM and GM base materials would lead to a shift of the measurement results obtained with, for example, real-time PCR.

The mass of DNA in both base materials was estimated using a slight modification of the classical fractionation method developed initially by Ogur and Rosen [11]. 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 performed. The mass of DNA was determined after derivatisation with diphenylamine using a spectrophotometer. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [11, 12]. 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 (1.023 ± 0.022) ($N = 9$ with an expanded uncertainty, $k = 2$). A t-test showed with 95 % confidence that no significant difference exists between the DNA mass extracted from the GM and non-GM soya powders using the modified Ogur and Rosen method.

The integrity of the DNA was assessed by capillary electrophoresis. DNA was extracted from 200 mg samples of the processed powder materials ERM-BF437a, ERM-BF437b, ERM-BF437c, ERM-BF437d and ERM-BF437e, using a CTAB DNA extraction method (Annex A). None of the samples showed DNA degradation (data not shown).

3.4 Confirmation measurements

As a control for the gravimetric preparations, the mass fraction of DAS-81419-2 soya in the mixed materials ERM-BF437c, ERM-BF437d and ERM-BF437e was confirmed using the real-time PCR method provided by Dow AgroSciences. This confidential method, targeting the transgenic DNA insertion in this soya and using a sample intake of 200 mg, was validated in-house. It will be published after validation by the European Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) [9].

The real-time PCR test was calibrated with genomic DNA extracted from pure DAS-81419-2 soya powder. At the IRMM, the genomic DNA was extracted by a validated CTAB extraction method (Annex A) using 200 mg powder samples. After the extraction, the DNA was diluted in a TE buffer solution (pH 8.0, 1 mmol/L Tris and 0.01 mmol/L EDTA) and used to produce calibration curves for the soya-specific gene and the transgene. For the calibration curve of the soya-specific gene, the DNA was used undiluted (approximately 200 ng DNA per 25 µL reaction) and diluted up to 200-fold. For the calibration curve of the transgene, the DNA was used undiluted (approximately 200 ng DNA per 25 µL reaction) and diluted up to 10000-fold. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of DAS-81419-2 and from the Cq-values. The LOD of the PCR method was taken as 3.3-fold s of the lowest calibration point at which s_{rel} was below 25 %. The results of the quantification of DAS-81419-2 are shown in Table 2. Quantification of the mass fraction of DAS-81419-2 in the powders by real-time PCR confirmed the consistency of the mass fractions for the gravimetrically mixed materials ERM-BF437c, ERM-BF437d and ERM-BF437e. However, as no independent calibration was carried out, the data in Table 2 can be used for confirmation of the processing, but do not necessarily represent the true value of the mass fractions.

Table 2: Quantification of the DAS-81419-2 soya mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure DAS-81419-2 seed powder for calibration

Candidate CRM	DAS-81419-2 soya mass fraction [g/kg]	U (k = 2) [g/kg]
ERM-BF437a	< 0.07 [1) 2)]	-
ERM-BF437b	1088 [1)]	82
ERM-BF437c	0.93 [3)]	0.05
ERM-BF437d	9.3 [4)]	0.5
ERM-BF437e	91 [1)]	4

1) Mean of 2 samples (extraction replicates) from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates.

2) The obtained value is below the LOD determined during method validation (0.07 g/kg).

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

4) Mean of 2 samples (extraction replicates) from each of 12 randomly selected bottles (N = 12, n = 2), with each sample measured in 3 real-time PCR replicates.

4 Homogeneity

A key requirement for any certified reference material aliquotted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty associated with the certified value, although it is not necessarily relevant whether the variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1] requires RM producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

This homogeneity study was planned together with the measurements to control the gravimetric preparations and the short-term stability of the CRMs (Sections 3.4 and 5.1). As the measurement results were obtained under intermediate precision conditions on bottles taken randomly from the entire batch and analysed in a randomised order they were as well suited to investigate the CRM homogeneity. Two extraction replicates per bottle were analysed for ERM BF437d and ERM-BF437e, compared to three for ERM BF437c. The number of extraction replicates was based on the intermediate precision of the in-house validated method, such that the standard uncertainty for the within-unit variation would be less than 25 %. Homogeneity of the blank powder was demonstrated by the test for the genetic purity of the raw materials (Section 3.1).

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the candidate CRMs were valid for all bottles containing the material, within the stated uncertainties.

For the between-unit homogeneity test, the number of bottles selected corresponds to approximately the cube root of the total number of bottles produced. Therefore, 12 bottles were selected for ERM-BF437c and 14 for ERM-BF437d. To facilitate both the homogeneity studies and the short-term stability study, 15 bottles were selected for ERM-BF437e. For each candidate CRM, a random stratified sampling scheme covering the whole batch was used to select the samples. For this, the batch was divided into 12, 14 and 15 groups respectively (with similar number of bottles) and one bottle was randomly selected from each group. For ERM-BF437c, three independent samples (extraction replicates) were taken from

each bottle, whilst for ERM-BF437d and ERM-BF437e, two independent samples (extraction replicates) were taken from each bottle. All samples were analysed by real-time PCR. Due to the number of PCR plates required, the measurements were performed under intermediate precision conditions. Samples were analysed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence. The results are shown in the figures in Annex B.

Regression analyses were performed to evaluate potential trends in the filling sequence. No trends were observed, to a 95 % confidence level.

In addition, regression analyses were performed to evaluate potential trends in the analytical sequence. No statistically significant trend was detected, to a 95% confidence level.

The dataset was assessed for consistency using single and double Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. No outlying individual results or outlying unit means were detected.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method intermediate precision, if the individual samples were representative of the whole unit.

Evaluation by ANOVA requires mean values per unit which follow at least a unimodal distribution, and results for each unit that have approximately the same standard deviation. The distribution of the mean values per unit was visually evaluated using histograms and normal probability plots. Too few data are available for the unit means to make a clear statement about the distribution. Therefore, the individual data were checked by eye to see if they followed a unimodal distribution using histograms and normal probability plots.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are therefore subject to random fluctuations. Therefore, the mean squares between groups ($MS_{between}$) can be smaller than the mean squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method intermediate precision, was calculated as described by Linsinger *et al.* [13]. u^*_{bb} is comparable to the LOD of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

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

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

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

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

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

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

Table 3: Results of the homogeneity studies

Candidate CRM	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]
ERM-BF437c	17.4	n.c.[1)]	5.4
ERM-BF437d	8.6	7.0	3.8
ERM-BF437e	8.9	3.0	3.8

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

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

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used 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 no longer contributes to analytical variation at this sample intake.

ERM-BF437a and ERM-BF437b are pure non-GM and GM materials, respectively. Therefore, the minimum sample intake for these materials is not linked to the within-unit homogeneity. Based on the real-time PCR measurements carried out on these two powders, it was concluded that 200 mg is a suitable minimum sample intake for real-time PCR based measurements.

5 Stability

Time, temperature and light were regarded as the most relevant influences on the stability of the materials. The influence of light was minimised by storing the materials in containers which minimised light exposure. In addition, materials were stored in the dark and dispatched in boxes, thus removing any possibility of light degradation. Therefore, only the influences of time and temperature needed to be investigated.

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The ERM-BF437e material was selected for the short-term stability study because it is a mixture of both GM and non-GM base materials, which makes it possible to assess the stability of each base material. The short-term stability study was carried out using an isochronous design [14]. In this approach, samples of ERM-BF437e were stored for a particular length of time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference

conditions). At the end of the isochronous storage, the samples are analysed simultaneously under intermediate precision conditions.

ERM-BF437 is a dried soya powder, which has been prepared in a similar manner to previous GMO CRM soya powders at the IRMM and which both have similar water content and particle size distribution. Therefore, the data obtained for the stability monitoring of previous soya GMO CRMs were used to estimate the uncertainty associated with storage for this CRM.

5.1 Short-term stability study

For the short-term stability study, units of ERM-BF437e were stored at 4 °C, 18 °C and 60 °C for each of 1, 2 and 5 weeks. Units representing the time point of 0 weeks were kept at a reference temperature of -70 °C. Five units per storage time and temperature were selected using a random stratified sampling scheme. From each unit, two extraction replicates were measured by real-time PCR. The measurements were performed under intermediate precision conditions with respect to the PCR plates, and a randomised sequence was used to differentiate any potential analytical drift from a trend over storage time.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No statistical outliers were detected for the analyte, and the results were retained for the estimation of u_{sts}.

In addition, the data were evaluated against storage time, and regression lines of mass fraction versus time were calculated, to test for potential increases/decreases of the DAS-81419-2 soya mass fraction due to shipping conditions. The slopes of the regression lines were tested for statistical significance. None of the trends were statistically significant at a 95 % confidence level for any of the temperatures.

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

The results of the measurements are shown in Annex C.

5.2 Long-term stability study

Data from the stability monitoring program for GMO CRMs were available. Previously released soya powder CRMs were analysed for their GM mass fraction on 16 occasions over a period of 6 years. At each occasion, measurements were performed simultaneously on one PCR plate, on units stored at normal storage temperature (4 °C) and at a reference temperature (-70 °C). Each of these studies can be viewed as a two-point isochronous study. The evaluation was based on the ratio of samples stored at 4 °C and -70 °C.

To verify that the data obtained from stability monitoring could be used to estimate the stability uncertainty contribution for ERM-BF437, an additional isochronous study was performed within the short-term stability assessment (Section 5.1). The data of the 4 °C short-term stability study did not contradict the conclusions drawn from the long-term stability study on the uncertainty contribution relating to the storage of the CRM.

The long-term stability data were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No statistical outliers were detected, and the results were retained for the estimation of u_{lts}.

In addition, the data were evaluated against storage time and regression lines were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to storage). No significant trend was detected at a 95 % confidence level.

The material can therefore be stored at 4 °C.

The results of the measurements are shown in Annex D.

5.3 Estimation of uncertainties

Due to the intrinsic variation of measurement results, no study can entirely rule out degradation of materials, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method intermediate precision, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation.

The uncertainties of stability during dispatch and storage were estimated, as described in [15]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

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

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

s_{rel} relative standard deviation of all results of the stability study
x_i result at time point *i*
$\bar{x}$ mean result for all time points
t_{tt} chosen transport time (1 week at 60 ºC)
t_{sl} chosen shelf life (24 months at 4 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the 60 °C studies. The uncertainty describes the possible change during a dispatch at 60 °C lasting for 1 week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the stability monitoring program for soya GMO CRMs. The uncertainty contribution describes the possible degradation during 24 months storage at 4 °C.

The results of these evaluations are summarised in Table 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 24 months.

Candidate CRM	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-BF437	0.7	1.3

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

6 Characterisation

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

This 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-BF437 are soya powder materials processed from non-GM and GM seeds. While ERM-BF437a is prepared from the pure blank material and ERM-BF437b from the pure GM material, the other candidate CRMs of the ERM-BF437 series are gravimetrically produced mixtures of the pure non-GM and GM seed powders. ERM-BF437 is certified for the mass fraction of DAS-81419-2 soya.

6.1 Genetic purity of the base materials

The genetic purity with respect to the DAS-81419-2 soya event of the GM and non-GM batches used for the processing of the powder candidate CRMs was investigated to calculate the certified value.

No indication was found that the GM soya material contained seeds that were negative for the event DAS-81419-2 (Section 3.1).

The powder used for the production of ERM-BF437a did not contain traces of DAS-81419-2 above the LOD of the real-time PCR method used (Sections 3.1 and 3.4). The certified value for ERM-BF436a is therefore based on the LOD of the real-time PCR method applied, as determined during in-house method validation.

Since no evidence of contamination was found in both base materials, 100 % genetic purity was used for the calculation of the certified mass fraction of DAS-81419-2 in the powder mixtures. The difference between the statistically established genetic purity of 98.6 % (Section 3.1) and the 100 % genetic purity was taken into account in the uncertainty calculation.

6.2 Mass fractions and their uncertainties

The certified mass values are based on the mass fractions of mixed GM and non-GM powder, corrected for their water mass fractions and taking into account the powder's genetic purity with regard to the DAS-81419-2 event. The values were calculated according to the following equations:

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

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

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

$m_{\text{GM,anhyd}}$ mass of the GM powder corrected for its water mass fraction
$m_{\text{nonGM,anhyd}}$ mass of the non-GM powder corrected for its water mass fraction
m_{GM} mass of the GM powder used for the dilution
m_{nonGM} mass of the non-GM powder used for the dilution
WMF_{GM} water mass fraction of the GM powder
$\text{WMF}_{\text{nonGM}}$ water mass fraction of the non-GM powder

In Table 5, the data supporting the calculation of the mass fractions of DAS-81419-2 soya are summarised.

Table 5: Subsequent mixing of GM DAS-81419-2 soya seed powder with non-GM powder to prepare the ERM-BF437c, d and e materials

Candidate CRM	GM powder [1)			Non-GM powder [1)		Mixtures
	Mass fraction [g/kg]	Water mass fraction $\pm U$ (k = 2) [g/kg]	Mass [g]	Water mass fraction $\pm U$ (k = 2) [g/kg]	Mass [g]	Calculated GM mass fraction [g/kg]
ERM-BF437e	1000.0 [2)	25.6 ± 1.6	398.6	25.9 ± 1.6	3600.2	100
ERM-BF437d	100.0 [3)	23.5 ± 1.5	398.0	25.9 ± 1.6	3600.8	9.9
ERM-BF437c	10.0 [4)	22.3 ± 1.4	397.4	25.9 ± 1.6	3601.4	0.99

[1) Calculations of the certified mass fraction of DAS-81419-2 soya in the powder mixtures are based on a 100 % genetic purity with regard to DAS-81419-2 of the non-GM and GM base materials.

[2) Pure GM powder ERM-BF437b was used for the preparation of ERM-BF437e.

[3) GM powder mixture ERM-BF437e was used for the preparation of ERM-BF437d.

[4) GM powder mixture of ERM-BF437d was used for the preparation of ERM-BF437c.

The uncertainties of the certified mass fractions (u_{char}) of DAS-81419-2 soya have several components, i.e. the uncertainty around the mass determination ($u_{char,1}$), the uncertainty around the water mass fraction analysis ($u_{char,2}$), and the uncertainties around the determination of the genetic purity with regard to the DAS-81419-2 event of the non-GM and GM base powders ($u_{char,3}$ and $u_{char,4}$, respectively). Based on a statistical analysis of the probability distribution of finding a negative seed in the GM base material, it was concluded that the genetic purity with regard to the event DAS-81419-2 was higher than 98.6 % (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-81419-2 soya in ERM-BF437

Candidate CRM	Nominal mass fraction [g/kg]	Standard uncertainty contribution [g/kg]				Combined standard uncertainty u_{char} [g/kg]
		$u_{char,1}$ [1)	$u_{char,2}$ [2)	$u_{char,3}$ [3)	$u_{char,4}$ [4)	
ERM-BF437a	0	n.a.[5)	n.a.[5)	0.0191	n.a.[5)	0.0191
ERM-BF437b	1000	n.a.[5)	n.a.[5)	n.a.[5)	3.9479	3.9479
ERM-BF437c	1	0.0053	0.0017	0.0191	0.0039	0.0202
ERM-BF437d	10	0.0432	0.0145	0.0191	0.0393	0.0631
ERM-BF437e	100	0.3057	0.1187	0.0191	0.3936	0.5127

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

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

[3) Standard uncertainty of the genetic purity estimation of the non-GM base material (LOD = 0.07 g/kg), based on the half-width of the interval between 0 and 0.07 g/kg, divided by the square root of 3 (rectangular distribution).

[4) Standard uncertainty of the genetic purity estimation of the GM base material (> 98.6 %), based on the interval between 98.6 % and 100 % divided by the square root of 3 (rectangular distribution).

[5) n.a.: not applicable

6.3 Verification measurements

Real-time PCR measurements demonstrated that no mixing errors were made (Section 3.4). Capillary electrophoresis proved that the DNA analyte was not degraded during processing of the candidate CRMs (Section 3.3).

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-GM seeds used in the gravimetrical preparation. The masses of the powders were corrected for their respective water mass fractions during the preparation of the materials (Table 5).

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} (Section 0.2), potential between-unit inhomogeneity, u_{bb} (Section 4.1), and potential degradation during transport, u_{sts}, and long-term storage, u_{lts} (Section 5.3). These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,rel}$) with a coverage factor k given as:

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

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

For the blank material, the LOD of the method was used to describe the 95 % confidence interval of the certified mass fraction of the event (< 0.07 g/kg). This was supported by the high genetic purity with regard the event DAS-81419-2 of the non-GM material and the absence of any mixing step; calculating the U_{CRM} for the blank material on the basis of the only quantifiable standard uncertainty ($u_{char,3}$) gives a value of U = 0.04 g/kg (assuming k = 2), which is below the certified < 0.07 g/kg value. The LOD is, therefore, a conservative estimate of the certified value and its uncertainty.

For the pure GM material, the statistically calculated genetic purity of the GM seed batch (Section 3.1) was used to describe the 95 % confidence interval of the certified mass fraction of the event (> 986 g/kg). Calculating U_{CRM} for the pure GM material on the basis of the only quantifiable standard uncertainty ($u_{char,4}$) gives a value of U = 8 g/kg (assuming k = 2), which is less than the difference between the nominal value (1000 g/kg) and the certified value (> 986 g/kg). The statistically calculated genetic purity is, therefore, a conservative estimate of the certified value and its uncertainty.

For the three mixtures, the certified values were established by gravimetry, and the measured mass values had an expanded uncertainty with a coverage factor of 2, established during calibration of the balance. Therefore, the same coverage factor (k = 2) was applied to obtain the expanded uncertainties for ERM-BF437c, d and e.

The certified values and their uncertainties are summarised in Table 7.

Table 7: Certified values and their uncertainties for ERM-BF437

Candidate CRM	Certified value [g/kg]	u_{char} [g/kg]	u_{bb} [g/kg]	u_{sts} [g/kg]	u_{lts} [g/kg]	U_{CRM} [3] [g/kg]
BF437a	< 0.07 [1]	0.0191	n.a.[4]	n.a.[4]	n.a.[4]	-
BF437b	> 986 [2]	3.9479	n.a.[4]	n.a.[4]	n.a.[4]	-
BF437c	0.99	0.0202	0.0536	0.0069	0.0129	0.12
BF437d	9.9	0.0631	0.6962	0.0696	0.1293	1.5
BF437e	100	0.5127	3.7889	0.6980	1.2962	9

[1] With 95 % confidence, the certified value is below this level.

[2] With 95 % confidence, the certified value is above this level.

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

[4] n.a.: not applicable

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The certified identity is based on the documentary traceability to the DAS-81419-2 application, submitted to the European Food Safety Authority (EFSA-GMO-NL-2013-116) [8].

Quantity value

The traceability chain for ERM-BF437c, d and e is based on the use of calibrated balances and a thorough control of the weighing procedure.

The certified values for the pure non-GM and GM CRMs ERM-BF437a and ERM-BF437b, respectively, are based on the genetic purity assessment using event-specific DAS-81419-2 soya real-time PCR.

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

8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent 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 that define this concept. For instance, the CLSI Guideline C53-A [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 is therefore a crucial characteristic when applying different measurement methods. When the 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 exist in calibration, nor can the CRM be used as a calibrant.

The candidate CRM is prepared from non-GM and GM soya seed powder and the analytical behaviour will be the same as for a routine sample of milled soya seeds/grains. 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 should be stored at (4 ± 3) °C in the dark. Care should be taken to avoid any change of the moisture content once the units are open, as the material is hygroscopic. The user should close any bottles immediately after taking a sample.

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

9.3 Minimum sample intake

The minimum sample intake for a DNA extraction is 200 mg soya powder.

ERM-BF437a and ERM-BF437b 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 for calibration or quality control of DAS-81419-2 soya detection methods. As with any reference material, they can be used for establishing control charts and validation studies.

The user is reminded that this reference material is certified for its DAS-81419-2 soya mass fraction and should be used for measurements expressed in mass fractions. The exact relationship between the certified GM powder mass fractions and the corresponding DNA copy number ratio is not known. Changing the measurement unit from mass fraction to copy number per haploid genome equivalent, for instance, requires the use of a conversion factor that is only an approximate value, thereby adding additional uncertainty to the measurement result.

Use as a calibrant

If this matrix material is used as calibrant, the uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty. Furthermore, it should be noted that using the same material for calibration and quality control limits the effectiveness of the control, as calibrant and quality control material are based on the same raw materials. If this is unavoidable, it is recommended that different mass fraction levels of ERM-BF437 are used for calibration and for quality control.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [17]).

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

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

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

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Annexes

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

<u>Solutions and reagents</u>

1) CTAB buffer A
   - 1 % (w/v) CTAB
   - 0.7 M NaCl
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA pH 8.0
2) CTAB buffer B
   - 1 % (w/v) CTAB
   - 0.1 M Tris-HCl, pH 8.0
   - 15 mM Na_2EDTA, pH 8.0
3) Chloroform:1-Octanol (24:1)
4) 1.2 M NaCl
5) 2-Propanol
6) 70 % Ethanol
7) TE low buffer
   - 1 mM Tris, pH 8.0
   - 0.01 mM Na_2EDTA, pH 8.0
8) Proteinase K, 20 mg/mL
9) RNase A, 100 mg/mL
10) 2-Mercaptoethanol
11) QIAGEN Genomic-tip 20/G columns (Qiagen, Venlo, NL)
12) Genomic DNA Buffer Set (including G2, QBT, QC and QF) (Qiagen, Venlo, NL)

<u>Protocol</u>

a) Weigh 200 mg powder in a 2 mL microcentrifuge tube.
b) Add 1.3 mL of CTAB buffer A + 5 µL RNase A + 6.5 µL proteinase K + 26 µL 2-mercaptoethanol and mix by vortexing.
c) Incubate 1 h at 65 °C, shaking at 1,400 rpm.
d) Centrifuge 10 min at 16000 x *g* at RT.
e) Transfer 750 µL of supernatant to a 2 mL microcentrifuge tube containing 1 mL of chloroform:1-octanol (24:1).
f) Mix thoroughly by inverting, incubate for 5 min at RT.
g) Centrifuge 10 min at 16000 x *g* at RT.
h) Transfer 600 µL of the supernatant to a new 2 mL microcentrifuge tube containing 700 µL of CTAB buffer B.

i) Mix thoroughly by inverting, incubate for 30 min at RT.

j) Centrifuge for 20 min at 16000 x *g* at RT.

k) Discard the supernatant by pipetting and conserve the pellet.

l) Add 200 µL of 1.2 M NaCl.

m) Incubate for 5 min at 50 °C, shaking at 1400 rpm.

n) Add 1.6 mL of G2 buffer + 2.5 µL of RNase A + 20 µL of proteinase K.

o) Incubate for 1 h at 50 °C, shaking at 500 rpm.

p) Centrifuge for 5 min at 16000 x *g* at RT.

q) Equilibrate a Qiagen Genomic-tip 20/G column with 1 mL of QBT buffer.

r) Apply the sample to the equilibrated Genomic-tip 20/G column.

s) Wash the genomic-tip 20/G column with 3 mL of QC buffer.

t) Elute the genomic DNA with 1 mL of QF buffer (pre-warmed at 50 °C) and collect the DNA in a 2 mL tube.

u) Add 700 µL of 2-propanol to each tube, invert 10 times.

v) Centrifuge for 30 min at 10000 x *g* at 4 °C, discard the supernatant by pipetting.

w) Wash the pellet with 1 mL of 70 % ethanol.

x) Centrifuge for 10 min at 13000 x *g* at 4 °C.

y) Discard the supernatant by pipetting and air-dry the pellet for about 10 min.

z) Dissolve the DNA pellet in 80 µL of TE low buffer preheated to 50 °C.

a1) Incubate for 10 min at 50 °C, shaking at 500 rpm.

a2) Let the pellet dissolve completely overnight at RT.

a3) Store at +4 °C (short term) or -20 °C (long term).

Annex B: Results of the homogeneity measurements

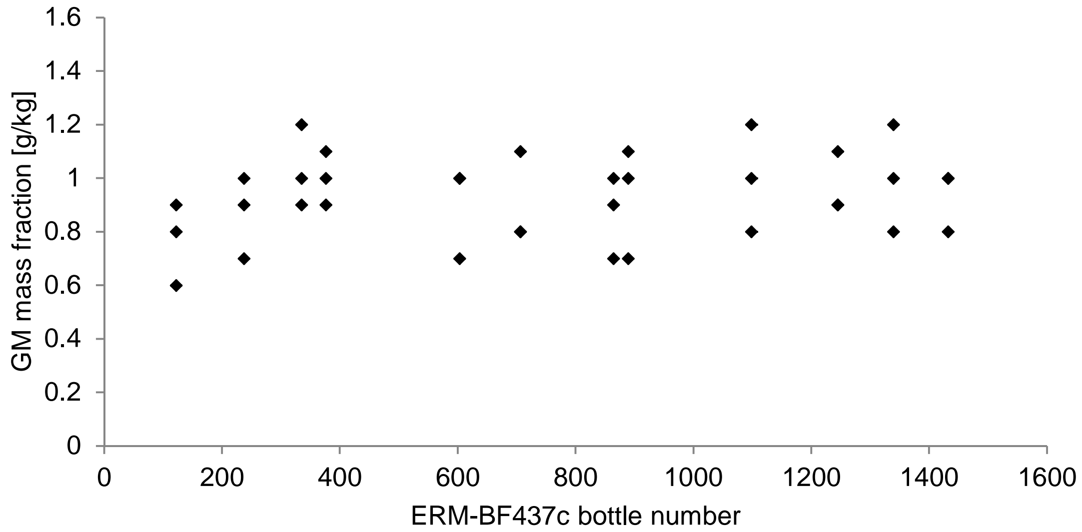

Figure B1: Real-time PCR measurement results obtained for ERM-BF437c. Three samples (extraction replicates) were measured from each of 12 randomly selected bottles (N = 12, n = 3), with each sample measured in 3 real-time PCR replicates.

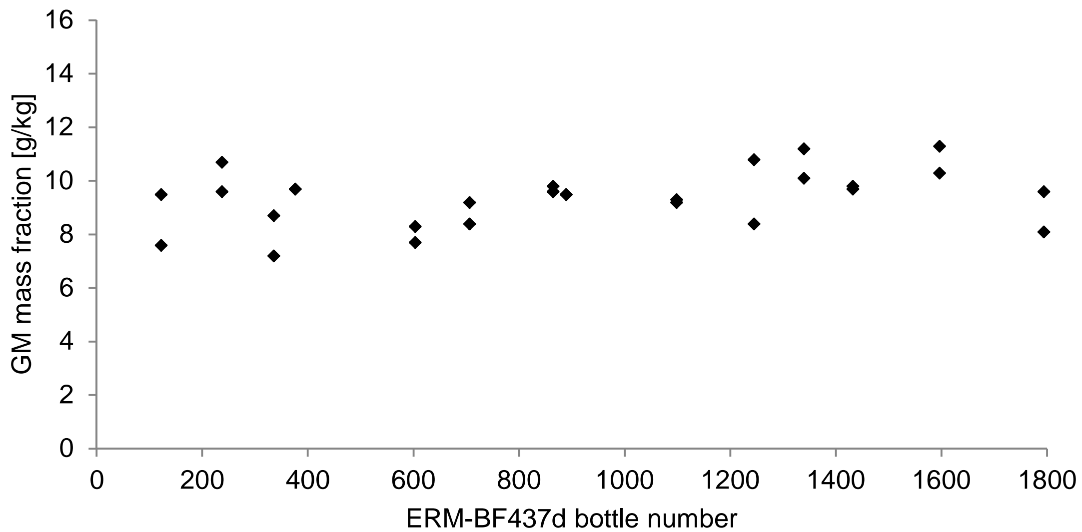

Figure B2: Real-time PCR measurement results obtained for ERM-BF437d. Two samples (extraction replicates) were measured from each of 14 randomly selected bottles (N = 14, n = 2), with each sample measured in 3 real-time PCR replicates.

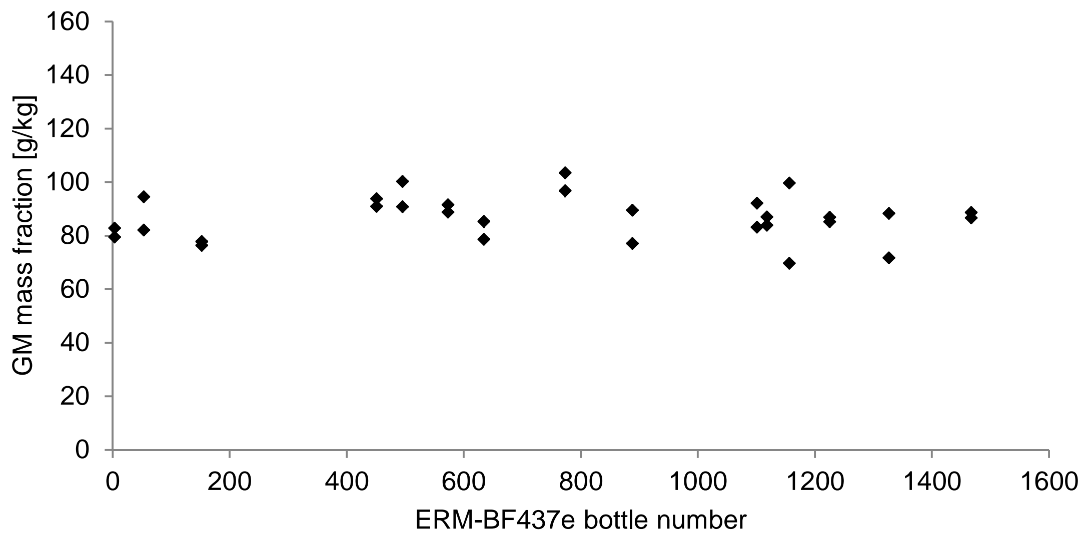

Figure B3: Real-time PCR measurement results obtained for ERM-BF437e. Three samples (extraction replicates) were measured from each of 15 randomly selected bottles (N = 15, n = 2), with each sample measured in 3 real-time PCR replicates.

Annex C: Results of the short-term stability measurements

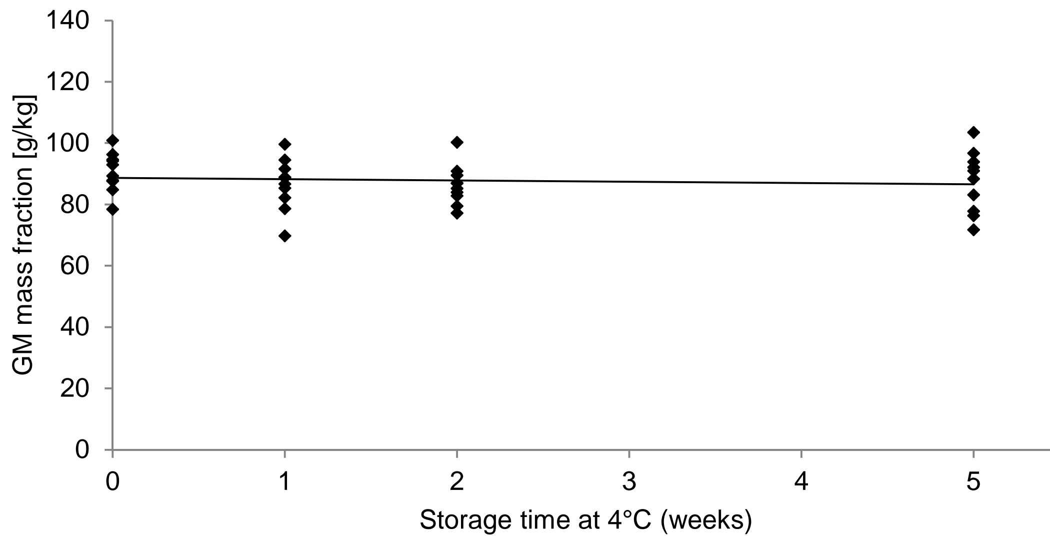

Figure C1: Real-time PCR measurement results obtained for ERM-BF437e during short-term stability testing at 4 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates. The fitted straight line obtained from a least-squares linear regression is shown.

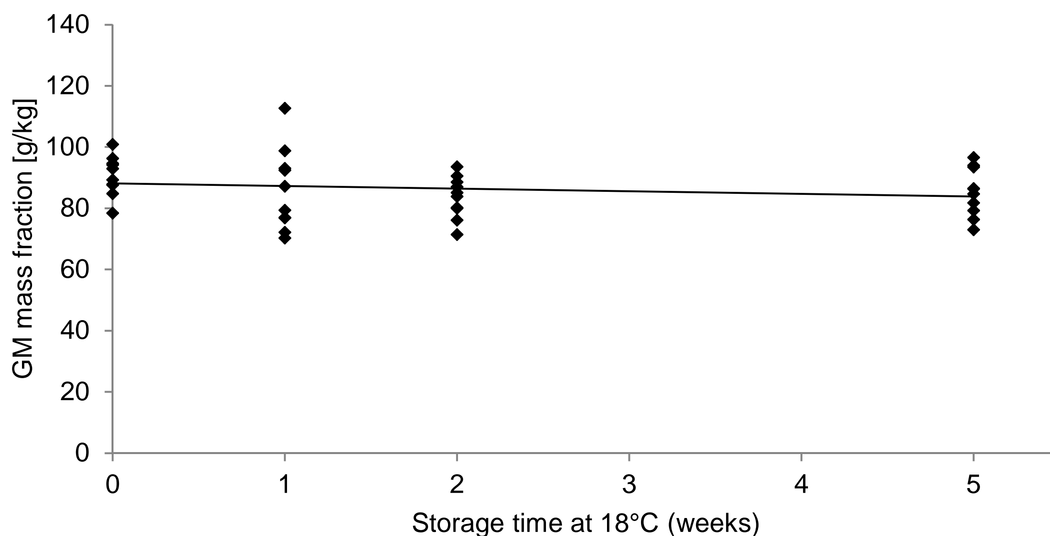

Figure C2: Real-time PCR measurement results obtained for ERM-BF437e during short-term stability testing at 18 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates. The fitted straight line obtained from a least-squares linear regression is shown.

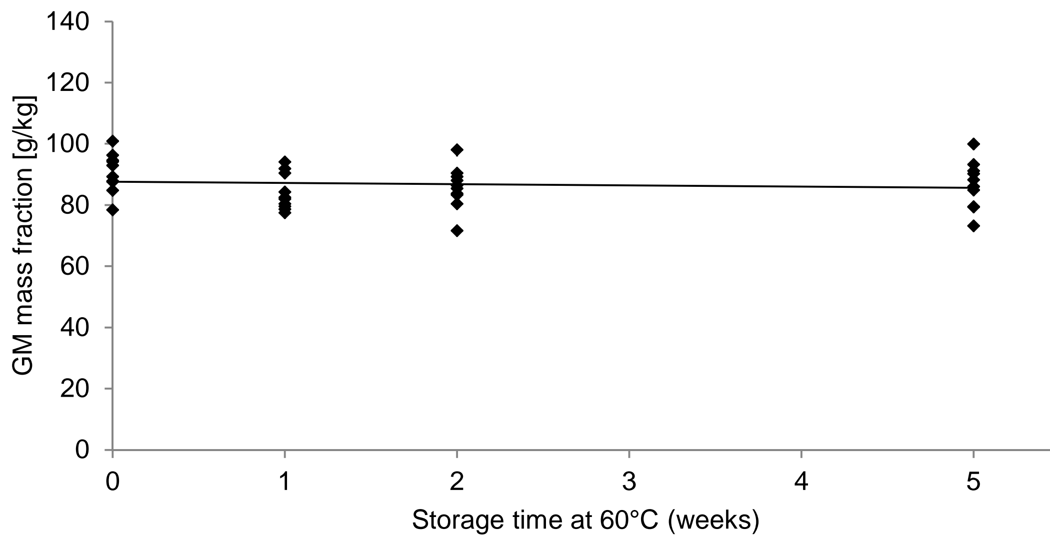

Figure C3: Real-time PCR measurement results obtained for ERM-BF437e during short-term stability testing at 60 °C. For each storage time, 2 samples (extraction replicates) were measured from each of 5 randomly selected bottles (N = 5, n = 2), with each sample measured in 3 real-time PCR replicates. The fitted straight line obtained from a least-squares linear regression is shown.

Annex D: Results of the long-term stability measurements

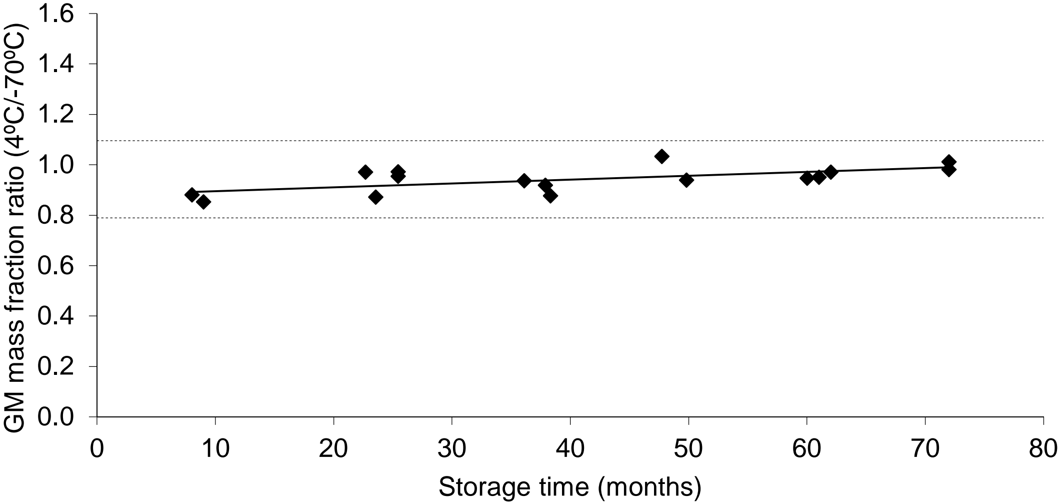

Figure D1: Real-time PCR measurement results obtained for ERM-BF410k, ERM-BF425, ERM-BF426 and ERM-BF432 during post-certification monitoring. The dashed lines give the limits of 3 *s* obtained for the measurement results. The fitted straight line obtained from a least-squares linear regression is shown.

European Commission

EUR 26869 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: **The certification of different mass fractions of DAS-81419-2 in soya seed powder Certified Reference Materials ERM®-BF437a, ERM®-BF437b, ERM®-BF437c, ERM®-BF437d and ERM®-BF437e**
Author(s): O. Zobell, A.M. Kortekaas, E. Scaravelli, M. Contreras, J. Seghers, M.-F. Tumba-Tshilumba, P. Corbisier, S. Trapmann
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Abstract
This report describes the production of a set of Certified Reference Materials (CRMs) ERM BF437a, b, c, d and e, which are certified for their DAS-81419-2 mass fractions. This material was produced following ISO Guide 34:2009 and is certified in accordance with ISO Guide 35:2006.
Genetically modified (GM) seeds of the soya event DAS-81419-2 and seeds from a non-GM soya variety were milled to obtain GM and non-GM seed powders with a similar particle size distribution. Mixtures of non-GM and GM soya powder were prepared gravimetrically.
The certified value was obtained from the gravimetric preparations, taking into account the genetic purity with respect to the event DAS-81419-2 of the two powder materials and their water mass fractions. The certified values were confirmed by event-specific real-time PCR as an independent verification method (measurements were within the scope of accreditation to ISO/IEC 17025:2005).
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) and include uncertainties relating to possible inhomogeneity (Section 4), instability (Section 5) and characterisation (Section 6).
The materials are intended for the calibration or quality control of real-time PCR measurements to identify DAS-81419-2 soya and/or quantify its mass fraction. As with any reference material, they can also be used for establishing control charts or for carrying out validation studies. The CRMs are available in glass vials containing at least 1 g of dried soya seed powder which were sealed under atmosphere of argon. The minimum amount of sample to be used is 200 mg.
The CRMs were accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

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