Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of Reference Materials of Soya Powder with different Mass Fractions of 356043 Soya

Certified Reference Materials ERM®-BF425 (ERM®-BF425a, ERM®-BF425b, ERM®-BF425c, ERM®-BF425d)

EUR 23029 EN - 2007

JRC EUROPEAN COMMISSION

The mission of IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
CRM Sales
European Commission
Joint Research Centre
Institute for Reference Materials and Measurements
Retieseweg 111
B-2440 Geel • Belgium
Email: jrc-irmm-rm-sales@ec.europa.eu
Tel.: +32 (0)14 571 705
Fax: +32 (0)14 590 406

http://www.erm-crm.org

EUR 23029 EN
ISBN 978-92-79-07464-6
ISSN 1018-5593
DOI 10.2787/2857

Luxembourg: Office for Official Publications of the European Communities

Printed in Belgium

CERTIFICATION REPORT

Certification of Reference Materials of Soya Powder with different Mass Fractions of 356043 Soya

Certified Reference Materials ERM®-BF425 (ERM®-BF425a, ERM®-BF425b, ERM®-BF425c, ERM®-BF425d)

D. Gancberg, J. Charoud-Got, P. Conneely, M. Contreras, P. Corbisier, S. Gioria, M. Van Nyen, S. Vincent, S. Trapmann

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

TABLE OF CONTENTS

GLOSSARY

als	acetolactate synthase encoding gene
ANOVA	Analysis of Variance
CRM	Certified Reference Material
CRL	Community Reference Laboratory
Ct-value	number of PCR cycles to pass a set threshold
df_{wb}	degrees of freedom within bottle
DNA	deoxyribonucleic acid
ERM®	trademark European Reference Materials
ELISA	Enzyme-Linked Immuno Sorbent Assay
FAM	6-carboxyfluorescein, fluorescent dye
*gat*4601	gene coding for an glyphosate acetyltransferase (GAT) protein and conferring resistance to the herbicide glyphosate
gDNA	genomic DNA
GM	genetically modified
GMO	genetically modified organism
hra	gene coding for the an acetolactate synthase (ALS) protein conferring tolerance to ALS-inhibiting herbicides
IRMM	Institute for Reference Materials and Measurements
JRC	Joint Research Centre
k	coverage factor
KFT	Karl Fischer titration
le1	soya-specific reference gene for GMO quantification, i.e. the single-copy *Glycine max* lectin gene *le1*
LOD	limit of detection
LOQ	limit of quantification
MS_{bb}	mean sum of squares between bottles
MS_{wb}	mean sum of squares within bottle
N	number of samples analysed
n	number of subsamples analysed
n.a.	not applicable
PCR	polymerase chain reaction
PSA	particle size analysis by laser diffraction
rt-PCR	real-time PCR
s	standard deviation
s_{bb}	standard deviation between bottles
SI	International System of Units
U	expanded uncertainty
u^*_{bb}	standard uncertainty component due to the inhomogeneity that can be hidden by method repeatability
u_{char}	standard uncertainty component due to the characterization of the material
u_{lts}	standard uncertainty component due to the long-term stability of the material
UV	ultra-violet
VIC	fluorescent dye
$\bar{x}$	average

1 Introduction

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) [1] that are authorised in accordance with Community legislation. In general, this demands on the one hand the development and validation of reliable GMO quantification methods, and on the other hand the production of reference materials for the quality control and calibration of these methods.

According to regulation (EC) No 65/2004 [2] the event 356043 soya corresponds to the unique identifier DP-356Ø43-5. The soya is genetically engineered to express two new genes: the *gat*4601 gene isolated from *Bacillus licheniformis* that confers tolerance to glyphosate herbicide and the *Glycine max hra* gene produced by modifying the *Glycine max als* gene that provides tolerance to acetolactate synthase-inhibiting herbicides.

The Institute for Reference Materials and Measurements (IRMM, Geel, BE) was asked by Pioneer Hi-Bred International Inc. to develop and produce a reference material for the quantification of 356043 soya. The major objective of the project was, therefore, the production of certified reference materials (CRMs) containing different mass fractions of the genetically modified 356043 soya seed.

2 CRM processing

2.1 Characterisation of the base materials

For the preparation of the CRMs, Pioneer Hi-Bred International Inc. (Johnston, IA, USA) supplied seeds of non-modified soya and GM 356043 soya to IRMM. Fifty kilogram of non-modified soya and 25 kg of 356043 soya were used for the processing of ERM®-BF425.
Pioneer Hi-Bred International Inc. carried out quality controls to assess the purity of the 356043 seed batch by testing 80 randomly chosen seeds for the presence of the transgene construct using an event-specific real-time PCR assay specifically designed for the detection of the 356043 soya. All 80 seeds were tested positive. From these data, the purity of the GM seed batch was estimated by Pioneer to be > 96.3 % at a 95 % confidence level. The purity of the non-GM seed batch was determined using the same event-specific PCR detection method for 356043 soya [3]. According to the supplier, approximately 300 ground seeds were collected from the non-GM seed batch and tested for the presence of the 356043 soya event (Table 1). All reactions were found negative. From these data, the purity of the non-GM seed batch was estimated by Pioneer to be > 99 % free of 356043 soya at a 95 % confidence level.

The purity and genetic composition of these batches were verified at IRMM by analysing genomic DNA (gDNA) extracted from leaves of seedlings in order to avoid influences from attached dust particles on the analytical results. Seeds of the non-GM and GM batches (N = 52 for each seed batch) were randomly chosen and allowed to germinate. Genomic DNA was extracted from pieces of the young leaves with a mass of approximately 100 mg for each plant using the QIAGEN Tissue Lyser and DNeasy® Plant Mini kit (Qiagen, Hilden, DE).
The extracted DNA was quantified using the PicoGreen® DNA quantification kit [4]. The quantitative rt-PCR was performed at IRMM using an ABI 7900HT instrument and primer pairs and labelled TaqMan® probes specific for the 356043 event or for the soya reference gene encoding the lectin gene *le1* [5], following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [6].

The results, summarised in Table 1, confirmed that all 52 plants from the GM batch contained the 356043 event. Similarly, all plants from the non-GM batch had a GM mass fraction below the limit of detection (0.5 g/kg). Statistical analysis (Poisson distribution for rare events) revealed that the non-GM and the GM soya seed batch both had a genetic seed purity > 94 % (95 % confidence level) with regard to the absence and presence of the 356043 event, respectively, in agreement with Pioneer's results.

Table 1: Genetic purity of the GM and non-GM seed batches used for the processing of ERM-BF425 with respect to GM genes

Batch	Test results reported by	Number of seeds tested	Number of GM positives	Number of GM negatives
Non-GMO	Pioneer	300[1)]	0	300[1)]
	IRMM	52	0	52
GMO	Pioneer	80	80	0
	IRMM	52	52	0

[1)] Two samples of 25 g (each sample representing approximately 150 seeds) were ground and homogenised; two DNA samples were prepared from 0.2 g of each of these two samples; 3 event-specific PCR reactions on each DNA sample were all negative for the GM event.

Additionally, the purity of the ground non-GM base material expressed as mass fraction of GM contamination was tested at IRMM. The analysis of randomly selected seeds and subsequent analysis of the powder (DNA extractions from three samples of 200 mg powder

using the GENE*Spin* kit, Eurofins, GeneScan GmbH, Freiburg, DE) indicated that no detectable GM contamination was found in the non-GM lot, i.e. the values obtained were all below the limit of detection (LOD) of the rt-PCR method applied.
Within the frame of an in-house validation of the method, the LOD and the limit of quantification (LOQ) were assessed. The LOD was calculated as (3.3*s*)/*b*, with *s* representing the standard deviation of the lowest GM mass fraction analysed and *b* the slope of the calibration curve. The efficiency of the amplification was determined based on the slope of the regression line between the GM mass fraction and the Ct-values, which should not be lower than the theoretical value of 3.322. The LOQ was calculated as (10*s*)/*b*. LOD and LOQ have been established by dilution of DNA extracted from pure GM 356043 powder in nuclease free water and were found to be 0.5 g/kg and 1.4 g/kg, respectively.

In conclusion, the purity analysis performed on the two seed powders (GM and non-GM) corroborated Pioneer Hi-Bred Inc. purity results. In addition, analyses were performed in order to verify the extractable DNA mass fraction in both powders using a slight modification of the classical fractionation method developed initially by Ogur & Rosen [7]. Indeed this parameter could influence the certified value of GM event in mixtures of GM and non-GM powders. A first step of removal of fatty compounds soluble in hexane was applied [8], followed by the sequential removal of alcohol-, alcohol-ether- and acid-soluble compounds and acidic extraction at 70 °C with 0.84 mol/L perchloric acid pH 0.3. The mass of ethanol-precipitating DNA was measured spectrophotometrically after derivatisation with diphenylamine. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [7, 9]. The ratio between the extractable DNA mass of the two materials was calculated as:

$$\frac{\text{Extractable mass of DNA in 100 mg 356043 soya powder}}{\text{Extractable mass of DNA in 100 mg nonGM soya powder}}$$

A DNA ratio of 1.07 ± 0.14 was found (Table 2), and a *t*-test demonstrated no significant difference at 95 % confidence level between the extractable mass of DNA of the two soya seed powders.

Table 2: Ratio of the precipitating DNA of GM and non-GM ground base material

Extraction method	***N***	**Mass fraction ratio** $\bar{x}$ ***± U (k = 2)***
Modified Ogur & Rosen [7]	9	1.07 ± 0.14

Using the modified Ogur & Rosen total DNA fractionation method, we could demonstrate that the extractable masses of DNA from non-GM and GM powders were similar.

2.2 Processing of the ground base materials

The GM and non-GM base materials were treated separately. Cross-contamination and contamination with foreign DNA were avoided using glove box systems and disposable laboratory clothing. All contact surfaces were treated with a DNA degrading solution prior to exposure to the base materials. An in-house validation study had proven beforehand, that the solution degrades DNA effectively under the given conditions.

The seeds processed were rinsed in demineralised water, drained, and dried under vacuum at 30 °C. This treatment led to a water mass fraction loss of approximately 45 g/kg in the case of the non-GM seeds and 51 g/kg in the case of the GM seeds (measured by volumetric KFT). The dried seeds were then ground using a high impact mill with a triangular ribbed open grinding track in order to obtain the ground base material. The high impact mill was flushed with nitrogen gas throughout the milling process. An additional

vacuum drying at 30 °C was carried out to further reduce the water content of the once ground base material by a mass fraction of approximately 40 g/kg. Then the dried-mixed powders were sieved through 0.5 mm sieves and the remaining fraction of particles milled for a second time. Slow feeding of the mill ensured that the whole base material passed the sieve and the different fractions were then Turbula-mixed altogether for 30-45 minutes. Care was also taken to avoid that the material was exposed to temperatures above 45 °C during mixing and milling. After measurement of the remaining water content, it was decided to carry out an additional vacuum drying at 30 °C. Finally, prior to gravimetric preparation of the GM and non-GM mixtures by dry-mixing, the twice-ground base materials had a water mass fraction of approximately 9 g/kg for the non-GM powder and 10 g/kg for the GM powder (measured by volumetric KFT, n = 3).

2.3 Gravimetric preparation of GM mixtures

The twice-ground base materials were used to produce powder mixtures containing mass fractions of 356043 soya seed powder at nominal levels of 0, 1, 10 and 100 g/kg. Prior to the dry-mixing, the remaining mass fractions of water in the ground GM and non-GM base materials were determined in triplicate by volumetric Karl Fischer titration (KFT, Metrohm, Berchem, BE) in order to correct the mass fractions for the water content of the ground base material. The mixture for the nominal mass fraction of 100 g/kg was produced first by mixing pure GM with non-GM ground base material. All lower mass fractions were achieved by serial dilution of the 100 g/kg GM powder with non-GM soya seed powder. Ground base materials were weighed using a calibrated balance with a relative standard uncertainty lower than 0.1 %. The powders were in a first step manually pre-mixed in a container and afterwards Turbula-mixed. The whole material was then transferred into a dry-mixing device and mixed for 2 minutes.

2.4 Bottling

The dry-mixed powders were bottled 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. Rubber stoppers were automatically placed on the vial neck. Before final closure of the vials the air was evacuated in a freeze-drier and replaced by argon. The vials were closed with the help of a hydraulic device in the freeze-drier 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 GM levels: nominal 0 g/kg - silver, nominal 1 g/kg - yellow, nominal 10 g/kg - red and nominal 100 g/kg - brown. For each concentration, labels were sticked onto each vial for identification (Figure 1). Subsequently randomised samples representative of the whole batch were selected.

Figure 1: Lay-out of the labels for the four CRMs. ERM-BF425a label was used for the non-GM material (blank), and ERM-BF425b, ERM-BF425c, and ERM-BF425d labels were used for a respective GM content of 1 g/kg (level 1), 10 g/kg (level 2) and 100 g/kg (level 3).

2.5 Processing control

The residual mass fraction of water was determined by volumetric KFT in ten randomly selected bottles from each of the powder mixtures and typically gave values in the interval of 11 to 20 g/kg (Table 3).

Table 3: Water mass fraction of the four CRMs (*N* = 10, *n* = 1)

CRM	Water mass fraction [g/kg]	
	$\bar{x}$	*s*
ERM-BF425a	11.3	2.9
ERM-BF425b	19.2	4.4
ERM-BF425c	16.9	2.5
ERM-BF425d	16.0	2.1

Five randomly selected bottles from each of the powder mixtures were used for particle size measurements with a particle size analyser based on laser diffraction (PSA, Sympatec, Clausthal-Zellerfeld, DE). From each bottle, 2 subsamples were analysed. The powders had a maximum particle size below 735 μm (Figure 2) and an average particle size around 120 μm, used for the calculation of the minimum sample intake (Section 3.2) and the calculation of the uncertainty budget (Section 5.3).

Additionally, a sieving test was carried out following ISO 3310-1 [10] using sieves with meshes of 45, 63, 90, 125, 180, 250, 500 and 710 µm (Figure 2) in order to confirm the data obtained with the particle size analysis. For sieving analysis the content of ten randomly selected bottles from each of the powder mixtures was merged to reach the required sample intake of 10 g. The maximum particle size of the materials was confirmed to be smaller than 710 µm.

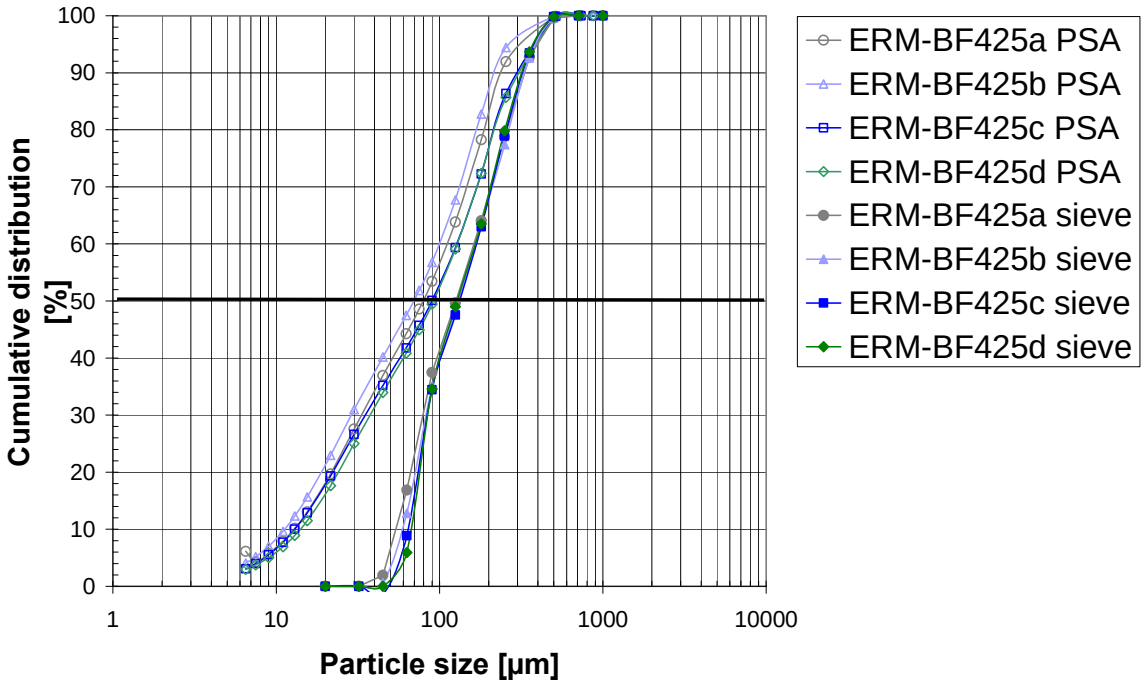

Figure 2: Average particle size distribution in ERM-BF425 by PSA (N = 5, n = 2) and sieving analysis (N = 1). The sieving data are cumulative mass fractions, the cumulative distribution of particles derived from laser light scattering data (PSA) is based on their equivalent volume diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles.

2.6 Confirmation of 356043 soya mixtures

The GM mass fractions of all four CRMs were confirmed using an event-specific rt-PCR method. During in-house validation, the LOD and LOQ of the method have been found to be 0.5 g/kg and 1.4 g/kg, respectively (for details see Section 2.1). The results obtained can be found in Table 4.

Table 4: Quantification by event-specific 356043 real-time PCR. DNA was extracted from 200 mg powder sample intakes using the GENE*Spin* kit (Eurofins GeneScan GmbH, Freiburg, DE). The measurements were calibrated with pure GM powder.

CRM	356043 mass fraction determined by event-specific rt-PCR [2) 3)] [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF425a	< 0.5 [1)]	n.a.
ERM-BF425b	1.0	0.3
ERM-BF425c	9.7	1.6
ERM-BF425d	96.9	14.7

[1)] The measured value was below the LOD of the method of 0.5 g/kg.
[2)] For each CRM the content of five randomly selected bottles was analysed and four subsamples (*N* = 5, *n* = 4) of each was measured in three replicates.
[3)] rt-PCR measures copy numbers of the targeted DNA sequence and was calibrated with known mass fractions of pure GM powder.

Results obtained with the event-specific rt-PCR method and being higher than the LOD (Table 4) were later compared to the certified values obtained from the gravimetrically prepared powder mass fractions (Section 5.3, Table 7). Quantification of the GM mass fraction of three mixtures of 356043 powders by rt-PCR proved to be consistent with the gravimetrically prepared mass fractions of CRM ERM-BF425. However, one has to be careful to draw quantitative conclusions (in gene copy number, for instance) from measurements of unknown samples as DNA- and/or protein-based GM quantification may vary with the particular matrix and soya variety tested [11].

3 Homogeneity

In order to ensure that the CRMs are sufficiently homogenous, two strategies were followed: validation of the mixing procedure and homogeneity control of the produced mixtures. The adequacy of the dry-mixing technology for the preparation of soya mixtures with different GM mass fractions has been shown previously using soya materials processed in the same way as described for the event 356043 soya [12]. Here we only report on the results of a homogeneity study performed on each of the event 356043 soya mixtures. Additionally, the recommended minimum sample intake is discussed.

3.1 Homogeneity study

The homogeneity of ERM-BF425 with respect to the event 356043 soya mass fraction was investigated by rt-PCR using bottles selected according to a random stratified procedure. The homogeneity of each of two CRMs, ERM-BF425c and ERM-BF425d, was investigated using 25 bottles that were analysed in random order under repeatability conditions using a sample intake of 200 mg powder and the GENE*Spin* kit DNA extraction method. As ERM-BF425b was processed and mixed in the same way as ERM-BF425c and ERM-BF425d, its homogeneity was assessed using 5 bottles only. Grubbs tests were performed to detect outlying individual results as well as bottle averages. No outliers were detected for any of the materials.

Regression analyses were used to evaluate potential drifts in results related to the analysis sequence or to the filling sequence. No significant trends were observed in the results.

It was furthermore checked whether the data followed a normal or unimodal distribution using normal probability plots and histograms respectively. The individual data and the bottle averages for all three CRMs were normally distributed.

ANOVA statistics were used to calculate the between bottles standard deviation (s_{bb}) and the maximum standard uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), using the formulas [13]:

$$s_{bb} = \sqrt{\frac{MS_{bb} - MS_{wb}}{n}} \qquad u^*_{bb} = \sqrt{\frac{MS_{wb}}{n}} \cdot \sqrt[4]{\frac{2}{df_{wb}}}$$

(MS_{bb} = mean sum of squares between bottles; MS_{wb} = mean sum of squares within bottles; n = number of replicates; df_{wb} = degrees of freedom within bottles)

Both values of s_{bb} and u^*_{bb} were converted into relative standard uncertainties and were expressed in percentage (Table 5). The largest of both values was included into the calculation of the overall uncertainty on the certified values (Section 5.3).
In order to determine the between bottles variation and the maximum hidden heterogeneity of CRM ERM-BF425, endogenous and event-specific PCR measurements on randomly selected bottles were carried out. For ERM-BF425b (nominal 1 g/kg) and ERM-BF425d (nominal 100 g/kg), the mean sum of squares between bottles was inferior to the mean sum of squares within bottle and it was calculated that the contribution to the homogeneity $u^*_{bb,rel}$ is smaller than 8.1 % and 3.3 %, respectively.

Comparison of the experimental data obtained during this homogeneity testing confirmed that the approach chosen for the estimation of the inhomogeneity uncertainty contribution (Section 5.3, Table 7) was valid.

Table 5: Standard uncertainties due to heterogeneity between bottles of dry-mixed event 356043 soya CRMs, analysed by rt-PCR using a sample intake of 200 mg

CRM	Number of samples analysed	$s_{bb, rel}$ [%]	$u^*_{bb, rel}$ [%]
ERM-BF425b	N = 5, n = 4	- [1)]	8.1
ERM-BF425c	N = 25, n = 4	4.5	3.6
ERM-BF425d	N = 25, n = 4	- [1)]	3.3

[1)] Using the ANOVA calculations, the mean sum of squares between bottles was inferior to the mean sum of squares within bottles, and the relative standard deviation between bottles homogeneity $s_{bb,rel}$ could not be calculated.

3.2 Minimum sample intake for analysis

Many commonly employed DNA extraction methods for plant powders recommend the use of 100 or 200 mg of powder as sample intake. A mass of 200 mg powder was employed throughout this certification project for DNA extraction by the GENE*Spin* method. The assumption that this amount of a substance of this size is representative for the whole material was investigated.

The mass density of the non-GM soya seed powder was determined by so-called tap-density measurements, carried out using the non-GM powder similarly to the procedure described in [14]. Taking into account the mass density (0.64 g/mL) and the particle size distribution (average particle size of 120 µm), it was calculated that the number of particles in a 200 mg sample is larger than 34×10^4. Consequently 200 mg of ERM-BF425b (nominal 1 g/kg) contain around 345 GM particles, supporting the assumption that the GM particles are well represented in 200 mg of sample.

As a general rule, it is advised to use sample intakes not smaller than 200 mg.

4 Stability

4.1 Short-term stability

In order to assess whether special care must be taken during transportation, a short-term stability of dried soya seed powder was investigated using an isochronous approach [15]. ERM-BF425c and ERM-BF425d were chosen for this study as they contained the highest GM mass fractions of the event 356043 soya CRMs (nominal 10 g/kg and nominal 100 g/kg respectively). Five bottles were stored at each of the Celsius temperatures 4 °C, 18 °C and 60 °C during 2, 4 and 8 weeks, and four subsamples from each bottle were analysed (N = 5, n = 4). A similar number of reference samples was likewise stored at -70 °C. Genomic DNA was extracted from the samples by the GENE*Spin* method, the extractable DNA content was determined by UV spectrometry and visualised by gel electrophoresis. No substantial DNA degradation was seen in any of the samples. Each DNA extract was analysed in triplicate by event-specific rt-PCR to reveal changes in GM quantification (Figure 3, nominal 100 g/kg and Figure 4; nominal 10 g/kg). Scrutinising the data obtained, no outliers (95 % confidence level) were detected by Grubbs tests. Regression analysis was performed for each of the storage temperatures to reveal any trend in GM quantity in relation to the time of storage. At both concentrations investigated, a *t*-test showed absence of trend after storage at 60 °C for a time period of 8 weeks (95 % confidence level). However, a trend was observed over the time period of 8 weeks for the nominal 100 g/kg soya seed powder (*t*-test, 95 % confidence level) for the samples stored at 18 °C, but not for those exposed to 4 °C or 60 °C. This trend was not detected after a time period of 4 weeks storage at 18 °C and at 60 °C. Therefore, it was concluded that the possibility of degradation during dispatch (always shorter than a 4 weeks period) is negligible for ERM-BF425c and ERM-BF425d. This statement can reasonably be extended to ERM-BF425a and ERM-BF425b given the similar composition of the four CRMs and the absence of influence of the GM/non-GM mass fraction ratios on the stability.
ERM-BF425 can be shipped under ambient conditions.

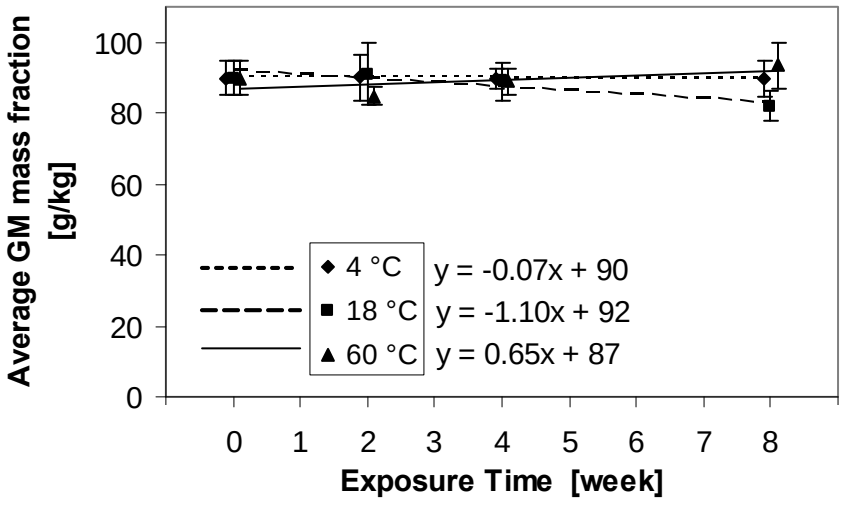

Figure 3: Short-term stability of ERM-BF425d stored at 4 °C (dotted regression line), 18 °C (dashed line) and 60 °C (solid line) for 2, 4 and 8 weeks and analysed by event-specific rt-PCR. The exposure time 0 week refers to the results obtained for samples stored at the -70 °C reference temperature during the 8-weeks study. The bars indicate the interval $\bar{X}$ ± s for N = 5; n = 4.

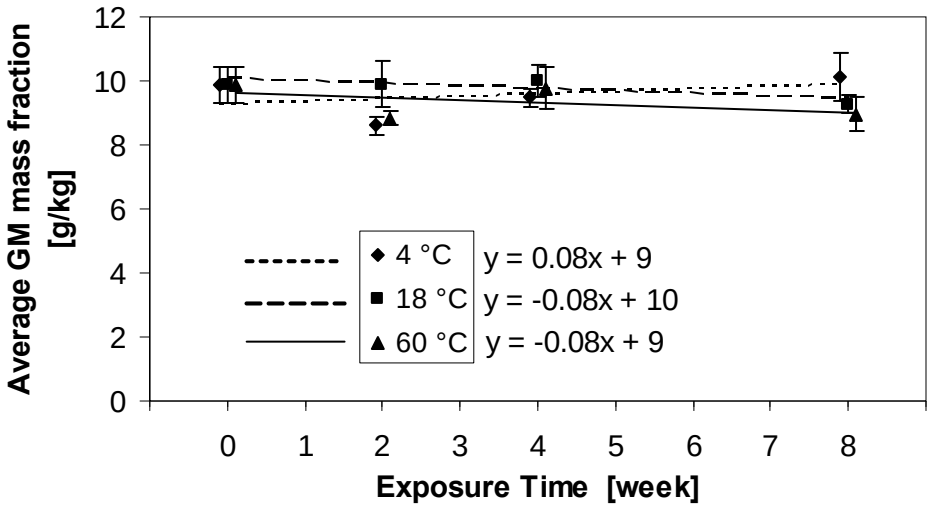

Figure 4: Short-term stability of ERM-BF425c stored at 4 °C (dotted regression line), 18 °C (dashed line) and 60 °C (solid line) for 2, 4 and 8 weeks and analysed by event-specific rt-PCR. The exposure time 0 week refers to the results obtained for samples stored at the -70 °C reference temperature during the 8-weeks study. The bars indicate the interval $\bar{x}$ ± *s* for *N* = 5; *n* = 4.

4.2 Long-term stability

The stability of the event 356043 soya seed powder was unaffected by short-term incubation at elevated temperatures (Section 4.1), similarly to what was observed for other soya matrices in the past [16]. There is also no reason to think that the event 356043 soya CRM would behave differently than other soya seeds CRMs processed the same way during long-term storage at 4 °C under controlled conditions. Therefore, it was decided to rely on IRMM's stability monitoring experience with soya seeds powder CRMs. The long-term stability of soya CRMs (ERM-BF410) during storage has been monitored at IRMM for a total of more than 5 years, using ELISA and/or event-specific rt-PCR methods (Figure 5, based on unpublished results). Although there was a visual trend in the stability data over the time period investigated, the statistical analysis revealed that this was not statistically significant (p = 0.15, *t*-test, 95 % confidence level). The relative standard uncertainty of the long-term stability (u_{lts}) [17], calculated from the available soya stability data, was approximately 2.2 % of the certified value per year and was used as the contribution due to instability of the CRMs upon storage in the total uncertainty budget (Table 7, Section 5.3).

In conclusion, the results demonstrate that the storage conditions of soya seed powder CRMs at IRMM are suited for long-term storage. In addition, post-certification monitoring is being carried out at regular intervals in order to check the stability of ERM-BF425.

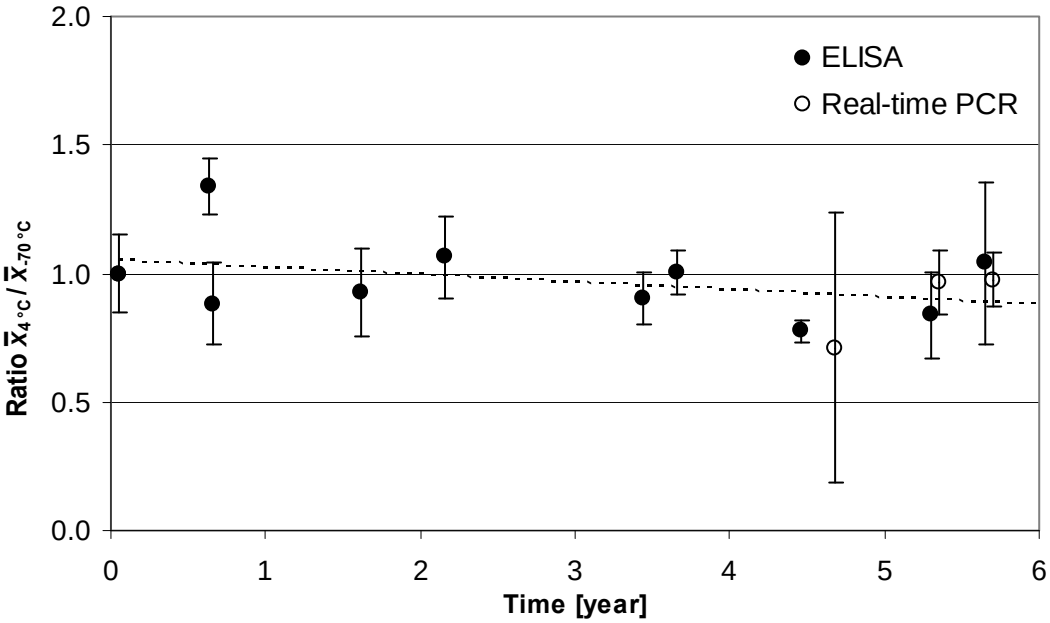

Figure 5: Long-term stability of dried soya seed powder (ERM-BF410, Roundup Ready soya) stored at 4 °C for various time periods, based on ELISA (●) and rt-PCR (○) measurements. The stability is expressed as the ratio between the GM mass fraction ratio in samples stored at 4 °C and that in samples stored for the same time period at the reference temperature (- 70 °C), with the bars indicating the expanded uncertainty interval ± *U* (*k* = 2). Each bullet corresponds to the average of 2 to 9 measurements. The dashed line is the regression line generated on the basis of all data points.

5 Certified mass fractions and uncertainty budgets

5.1 Metrological traceability

ERM-BF425a, ERM-BF425b, ERM-BF425c and ERM-BF425d are four reference materials certified for the mass fraction of 356043 soya seed powder. The certified mass fractions are based on gravimetric dry-mixing of non-modified soya seed powder with 356043 soya seed powder.

The certified value is traceable to the SI. The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure. The purity of the used seeds has been taken into account when calculating the certified value.

The user of the certified reference material should, however, bear in mind that the GM copy number ratio measured by rt-PCR could potentially differ from the values of certified GM mass fractions as a result of different DNA extraction efficiencies from GM and non-GM powders.

5.2 Certified value

The four CRMs under the label ERM-BF425 are soya seed powder CRMs processed from pure non-GM and pure GM base materials. While ERM-BF425a is prepared from the pure non-GM blank material, ERM-BF425b, c, d are gravimetrically produced mixtures of the pure non-GM and GM powders. The certified value is based on the GM mass fraction of dry-mixed GM and non-GM powder, corrected for their water mass fractions, and taking into account the powder's purity with regard to the 356043 event. If we assume that the purity of the non-GM powder is 100 %, which was supported by the data, the GM mass fractions can be calculated according to the following formulas:

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

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

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

(p_{GM} = purity of the GM powder used for the dilution; WMF = Water Mass Fraction; anhyd = anhydrous)

In Table 6, the data supporting the calculation of the certified values are summarised.

Table 6: Characterisation of ERM-BF425 based on gravimetry

ERM	GM powder			Non-GM powder			Certified GM mass fraction [g/kg]
	Genetic purity [g/kg]	Water mass fraction [g/kg]	Weighed powder mass [g]	Genetic purity [g/kg]	Water mass fraction [g/kg]	Weighed powder mass [g]	
BF425d	1000	9.8	400.33	1000	8.9	3599.67	100
BF425c	100.0 [1)]	9.2	400.11	1000	8.9	3599.89	10.0
BF425b	10.0 [2)]	7.5	399.49	1000	8.9	3600.51	1.0
BF425a	n.a.	n.a.	0	1000	8.9	4000.00	< 0.5 [3)]

[1)] For the preparation of BF425c, the nominal 100 g/kg GM soya was used.
[2)] For the preparation of BF425b, the nominal 10 g/kg GM soya was used.
[3)] Based on the LOD of the method.

For the purity of the GM base material the genetic identity of randomly selected seeds has been checked (Section 2.1). No evidence of the occurrence of non-GM seeds among the GM seeds was found (data from Pioneer and IRMM). Based on a statistical analysis of the distribution of the probability to find a negative seed in the GM base material, it could be concluded that the purity was higher than 96 % (95 % confidence level). For the calculation of the certified value, a GM purity of the seed batch of 100 % was used, based on the actual number of positive seeds detected per total number of seeds analysed (80 out of 80 seeds tested). Similarly, the non-GM impurity was taken as 0 % as no contamination was found during analysis of the corresponding seed batch.

5.3 Uncertainty budget

Controlled processing techniques in combination with purity controls of the GM and non-GM seeds and the derived base materials allow certifying the GM mass fractions in the CRMs with relatively low uncertainties (Table 7).

The combined standard uncertainty of the certified value comprises contributions from the between-bottle inhomogeneity at the recommended sample intake of 200 mg (u^*_{bb}), the long-term stability of the material (u_{lts}, calculated for 12 months) and the characterisation of the materials (u_{char}). The u_{char} includes uncertainties related to the weighing procedure, the determination of the water mass fraction in the powders, and the analysis of the purity of non-GM and GM base materials (Table 7). To calculate the expanded uncertainty corresponding to a 95 % level of confidence a coverage factor of 2 was used [18].

For the blank material, the LOD of the method (and not the LOQ) was used to describe the 95 % confidence interval on the certified value (< 0.5 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 0.2 g/kg, which is below the certified < 0.5 g/kg.

The uncertainty introduced by the inhomogeneity has been estimated on the basis of the heterogeneity of a Poisson distributed sample. The uncertainty contribution of the long-term stability testing has been estimated by monitoring nominal 10 g/kg soya seed powder CRMs by ELISA or rtPCR over time (Figure 5). A coverage factor of 2 was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % (Table 7).

Table 7: Uncertainty budget for the mass fraction of 356043 soya in ERM-BF425

ERM	Certified value [g/kg]	Standard uncertainty contributions [g/kg]						Expanded uncertainty U (k = 2) [g/kg]
		u_{bb} or s_{bb} 2)	u_{lts} 3)	$u_{char,1}$ 4)	$u_{char,2}$ 5)	$u_{char,3}$ 6)	$u_{char,4}$ 7)	
BF425a	**< 0.5** 1)	n.a.	n.a.	n.a.	n.a.	0.1357	n.a.	-
BF425b	**1.0**	0.0810	0.0225	0.0013	0.0001	0.1357	0.0108	0.4
BF425c	**10.0**	0.4500	0.2245	0.0131	0.0005	0.1357	0.1081	1.1
BF425d	**100**	3.3000	2.2451	0.0924	0.0043	0.1357	1.0807	9

1) With a 95 % probability, the certified value is below this level. The LOD was used to calculate the certified property of the blank material according to the GUM [19].
2) Standard uncertainty contribution resulting from the homogeneity assessment (Table 5).
3) Standard uncertainty resulting from the stability of dried soya seed powders during storage, extrapolated to 12 months.
4) Standard uncertainty of the mass determination mainly based on the uncertainty of the balance and the number of weighing steps required.
5) Standard uncertainty of the water mass fraction determination by volumetric KFT, based on the standard uncertainty of the method (1.6 g/kg) and the highest water mass fraction found in any of the powders used for mixing (19.4 g/kg for the non-GM powder).
6) Standard uncertainty introduced by the purity of the non-GM base material (LOD = 0.5 g/kg), based on the half-width of the interval between 0 and 0.5 g/kg, divided by the square root of 3 (rectangular distribution).
7) Standard uncertainty introduced by the purity of the GM base material (> 96 %), based on the half-width of the interval between 96 % and 100 % divided by the square root of 3 (rectangular distribution).

6 Intended use and instructions for use

ERM-BF425a, b, c, d are intended for use as quality control materials or calibrants in DNA- or protein-based methods for the detection of genetically modified material in food and feed.

The recommended minimum sample intake is 200 mg.

The materials are hygroscopic. Bottles should be stored dry and in the dark at maximum 4 °C. The user is advised to close bottles immediately after taking a sample for analysis.

7 References and acknowledgements

References

[1] Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC, Official Journal of the European Union, L 268/24.

[2] Commission Regulation (EC) No 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms, Official Journal of the European Union, L10/5.

[3] JRC/CRL website: http://gmo-crl.jrc.it//statusofdoss.htm.

[4] Molecular Probes (2005): Handbook of Fluorescent Probes and Research Products, Section 8.3: Nucleic Acid Detection and Quantitation in Solution, http://probes.invitrogen.com/handbook.

[5] Protocol for event-specific quantitation of DP-356Ø43-1 soybean. Should become accessible on the homepage of the Community Reference Laboratory for GM Food and Feed (http://gmo-crl.jrc.it/statusofdoss.htm) after validation of the method.

[6] TaqMan® Universal PCR Master Mix protocol, Applied Biosystems. See website http://docs.appliedbiosystems.com/pebiodocs/04304449.pdf.

[7] Ogur M., Rosen G. (1950): The Nucleic Acids of Plant Tissues. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch Biochem 25:262-276.

[8] Terry C.F., Harris N., Parkes H.C. Detection of genetically modified crops and their derivatives: critical steps in sample preparation and extraction. J. AOAC International (2002) 58: 768-774.

[9] Ganguli P.K. (1970): A sensitive procedure for the estimation of deoxyribonucleic acid by the diphenylamine reaction in the presence of cupric sulphate, Rev. Can. Biol. 29, 339-346.

[10] ISO 3310-1: (2000). Test sieves – Technical requirements and testing. Part 1: Test sieves of metal wire cloth.

[11] Website www.erm-crm.org, Application Note 4: Use of Certified Reference Materials for the quantification of GMO in food and feed.

[12] Trapmann S., Catalani P., Conneely P., Corbisier P., Gancberg D., Hannes E., Le Guern, L., Kramer, G.N., Prokisch, J., Robouch, P., Schimmel H., Zeleny, R., Pauwels, J, Van den Eede, G., Weighardt, F., Mazzara, M, Anklam, E. (2004) The Certification of Reference Materials of Dry-Mixed Soya seed powder with different Mass Fractions of Roundup Ready™ soya. EC certification report EUR 20273 EN.

[13] Linsinger T.P.J., Pauwels J., van der Veen A.M.H., Schimmel H., Lamberty A. (2001) Homogeneity and stability of reference materials. Accred. Qual. Assur. 6:20-25.

[14] ISO/IEC 3953 (1993) Metallic powders - Determination of tap density.

[15] Lamberty A., Schimmel H., Pauwels J. (1998): The study of the stability of reference materials by isochronous measurements, Fresenius J. Anal. Chem. 360:359-361.

[16] Trapmann S., Catalani P., Conneely P., Corbisier P., Gancberg D., Hannes E, Le Guern L., Kramer G.N., Prokisch J., Robouch P., Schimmel H., Zeleny R., Pauwels J., Van den Eede G., Weighardt F., Mazzara M., Anklam E. (2004) The Certification of Reference Materials of Dry-Mixed Soya seed powder with different Mass Fractions of Roundup Ready Soya. Certified Reference Materials ERM-BF410. EC certification report EUR 20273 EN, ISBN 92-894-3725-1.

[17] ISO (1995): Guide to the Expression of Uncertainty in Measurement, ISBN 9267-101889.

[18] Linsinger T.P., Pauwels J., Lamberty A., Schimmel H.G., van der Veen A.L., Siekmann L. (2001) Estimating the uncertainty of stability for matrix CRMs. Fresenius J Anal Chem 370:183-188.

[19] EURACHEM / CITAC Guide (2000) Quantifying Uncertainty in Analytical Measurement. See http://www.measurementuncertainty.org/mu/guide/index.html.

Acknowledgements

The authors acknowledge the contributions from K. Teipel for technical assistance, B. Toussaint and J. Charoud-Got (IRMM) for the reviewing of the certification report, as well as the experts of the Certification Advisory Panel 'Biological Macromolecules and Biological / Biochemical Parameters', R. Dybkaer (Frederiksberg Hospital, DK), E. Jansen (National Institute for Public Health and the Environment, NL) and U. Örnemark (LGC Standards, SE) for their critical comments.

European Commission

EUR 23029 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of Reference Materials of Soya Powder with different Mass Fractions of 356043 Soya, ERM®-BF425 (ERM®-BF425a, ERM®-BF425b, ERM®-BF425c, ERM®-BF425d)
Author(s): D. Gancberg, J. Charoud-Got, P. Conneely, M. Contreras, P. Corbisier, S. Gioria, M. Van Nyen, S. Vincent, S. Trapmann
Luxembourg: Office for Official Publications of the European Communities
2007 – 20 pp. – 21.0 x 29.7 cm
EUR - Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-07464-6

Abstract
This report describes the preparation and certification of soya seed powder CRMs with different mass fractions of genetically modified 356043 soya seed powder. After a two step grinding process, transforming the seeds into a non-modified soya seed powder and a 356043 GM soya seed powder, the mixtures, containing different mass fractions of GM, were gravimetrically prepared by dry-mixing. The CRMs are intended for the quality control and calibration of methods for the detection of genetically modified food and feed. The certified 356043 mass fractions of ERM®-BF425 were confirmed by event-specific real-time PCR as independent verification method.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

JRC
EUROPEAN COMMISSION

LA-NA-23029-EN-C

ISBN 978-92-79-07464-6

9 789279 074646

Publications Office
Publications.eu.int