Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of Reference Materials of Soya Seed Powder with different Mass Fractions of Genetically Modified 305423 Soya

Certified Reference Materials ERM®-BF426 (ERM®-BF426a, ERM®-BF426b, ERM®-BF426c, ERM®-BF426d)

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TABLE OF CONTENTS

GLOSSARY

305423 soya	GM soya (*Glycine max* (L.) Merr.) event 305423 containing the *Glycine max* genes *fad2-1*, coding for a D12-desaturase, and *hra*, providing herbicide resistance
ANOVA	analysis of variance
b	slope in the equation of linear regression $y = a + bx$
CRM	Certified Reference Material
Ct-value	number of PCR cycles to pass a set cycle threshold
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERM®	trademark of European Reference Materials
GM	genetically modified
GMO	genetically modified organism
IRMM	Institute for Reference Materials and Measurements
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
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
TaqMan®	*Thermus aquaticus* (Taq) DNA polymerase-based technology for fluorescent signal generation during rt-PCR
T-DNA	transfer DNA, i.e. the transgenes-containing DNA fragment transferred to the plant during *Agrobacterium*-mediated genetic transformation
U	expanded uncertainty
u_{bb}	standard uncertainty related to the between-bottle heterogeneity
u^*_{bb}	standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
u_{char}	standard uncertainty related to the characterisation
u_{lts}	standard uncertainty related to the long-term stability of the material
$\bar{x}$	average

1 Introduction and design of the project

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]. This is the labelling threshold level for GMOs 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.

Pioneer Hi-Bred International, Inc. (Johnston, IA, USA) has developed the genetically modified (GM) soya event 305423. Following Commission Regulation (EC) No 65/2004 [2], the 305423 soya received the unique identifier code DP-3Ø5423-1. The *Agrobacterium*-mediated genetic modification introduced a second copy of the *Glycine max* gene *fad2-1*, encoding a D12-desaturase, under control of a seed-specific promoter. As a result, both the introduced GM gene and the endogenous gene are silenced, i.e. their coding into the corresponding desaturase enzyme is prevented. Consequently, the conversion of oleic acid to linoleic acid, which is otherwise catalysed by this enzyme, is impaired. The GM soya, therefore, produces beans with an elevated level of (mono-unsaturated) oleic acid and decreased levels of (poly-unsaturated) palmitic, linolenic and linoleic acids. The oil extracted from this GM soya is more heat-stable than that from conventional soya and may be used directly in deep frying applications without the need for chemical hydrogenation to convert the poly-unsaturated fatty acids to trans- or saturated fatty acids. The 305423 soya also harbours the *hra* gene, an optimised form of the soya *als* gene, providing tolerance to acetolactate synthase-inhibiting herbicides.

The Institute for Reference Materials and Measurements (IRMM, Geel, BE) was asked by Pioneer Hi-Bred International to develop and produce a reference material for the quantification of 305423 soya. The major objective of the project was, therefore, the production of certified reference materials (CRMs) containing different mass fractions of the genetically modified 305423 soya seed.

2 CRM processing

2.1 Characterisation of the base materials

For the preparation of the CRMs, Pioneer Hi-Bred International, Inc. supplied non-modified soya seeds and 305423 soya seeds to IRMM. The 305423 soya seeds are homozygous GM seeds resulting from several cycles of self-fertilisation of a transgenic line harbouring a single copy of the GM insertion. The non-GM comparator line is the parental cultivar used for the genetic transformation and is itself a commercial cultivar resulting from conventional breeding (note that the same non-GM seed lot was also used for the production of ERM-BF425, a GMO CRM for the detection of another GM event developed by the same company). Quality control was done on both seed lots by Pioneer as follows: for the GM seed lot, 80 individual seeds were analysed for the 305423 event by real-time PCR (rt-PCR) and all tested positive (Table 1). This corresponds to an estimated GMO purity of > 96.3 % (95 % confidence level). For the non-GM seed lot, two samples of 600 seeds were homogenised, then a 16 g subsample from each ground seed sample was used for a single DNA extraction, and the two DNA extracts were tested in triplicate for the presence of the 305423 event. As all reactions were negative for this event (Table 1), Pioneer concluded with 95 % confidence that the non-GM seed lot was > 99.8 % free of 305423 soya.

The reported purity and genetic composition of both soya seed batches were verified at IRMM by analysing 50 randomly selected GM seeds and 50 randomly selected non-GM seeds for the presence of the GM event 305423. In order to avoid influences from attached dust particles on the analytical results, seedlings were grown and genomic DNA was extracted from the leaves. Quantitative rt-PCR was performed using primer pairs and labelled TaqMan® probes specific for the 305423 event or for the soya reference gene encoding the lectin gene *le1* [3]. Detection was done on an ABI7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [4]. The results, summarised in Table 1, confirmed that all 50 plants from the GM batch contained the 305423 event. Similarly, all plants from the non-GM batch had a GM mass fraction ratio below the limit of detection (0.8 g/kg). Statistical analysis (Poisson distribution for rare events) revealed that the non-GM and the GM soya seed batch both had a genetic purity > 94 % (95 % confidence level) with regard to the absence and presence of the 305423 event respectively, confirming the companies' results.

Table 1: Genetic purity of the non-GM and GM seed batches used for the processing of ERM-BF426 with respect to GM event 305423

Batch	Test results reported by	Number of seeds tested	Number of GM positives	Number of GM negatives
Non-GMO	Pioneer	1200[1)]	0	1200[1)]
	IRMM	50	0	50
GMO	Pioneer	80	80	0
	IRMM	50	50	0

[1)] Two samples of 600 seeds were ground and homogenised; DNA samples were prepared from 16 g of each of these powders; 3 event-specific rt-PCR reactions on each DNA sample were all negative for the GM event.

After arrival, the soya seeds were stored at 4 °C in the dark until use. Twenty five kg of non-GM soya seeds and ten kg of 305423 soya seeds were used for the processing of ERM-BF426.

2.2 Production of the base materials

The GM and non-GM base materials were processed separately. Cross-contamination and contamination with foreign DNA were avoided using glove box systems and clean 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 degraded DNA effectively under the given conditions. If required, the base powders were stored for short time periods in closed plastic containers.

The soya beans were rinsed in demineralised water, drained, and dried under vacuum at 30 °C. This resulted in a water mass fraction loss of approximately 45 g/kg for both seed batches (measured by volumetric KFT). The dried seeds were then milled 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 and milling was interrupted if the temperature rose above 40 °C. An additional vacuum drying at 30 °C was carried out to further reduce the water content of the once ground base material. The final water mass fraction in the non-GM and GM base materials was 7.8 g/kg and 7.7 g/kg respectively (n = 3; measured by volumetric KFT). Each ground base material was mixed in a turbula mixer for 30 minutes to improve equal distribution of the different parts of the soya tissues separated by the milling process. The powders were then stored in closed plastic containers.

2.3 Gravimetric preparation of GM mixtures

The ground base materials were used to produce a GMO blank material and three powder mixtures containing mass fractions of 305423 soya seed powder in non-GM soya seed powder at nominal levels of 5, 10 and 100 g/kg. All four materials, including the blank powder, were treated according to the same procedure. The powder materials were weighed using a calibrated balance with a relative standard uncertainty lower than 0.1 %. The masses of non-GM and GM soya powders required to produce the mixtures were corrected for the water mass fraction of the starting materials. The starting materials were combined in one container, turbula-mixed for 30 min, and further mixed in a special dry-mixing device for another 2 min. The blank material was processed first, followed by the mixtures. The nominal mass fraction of 100 g/kg was produced by mixing pure GM with pure non-GM ground base materials. The 10 g/kg mass fraction was produced by further dilution of the 100 g/kg GM powder with pure non-GM powder, and the 5 g/kg was likewise produced by further diluting the 10 g/kg mixture with non-GM powder.

2.4 Additional milling of the powders

Due to the remaining presence of some coarse particles in the once-milled powders, an additional milling was done for improved DNA extractability. This second milling was done after the dry-mixing of the GM and non-GM base materials (Section 2.3) because experience showed that the powders resulting from a double milling are rather sticky and hence more difficult to mix during the preparation of the gravimetrical mixtures. For this reason, the coarse particles were first sieved out of the blank and the mixed powders, milled as before (Section 2.2), and then added back to the original main batch. Sieving was done in an industrial sieving machine using a sieve with a mesh of 500 µm, starting with the non-GM powder and followed by the nominal 5, 10 and 100 g/kg powders. The twice milled fraction corresponded to approximately 12 % of the mass of each of the powders. A further turbula-mixing of the powders during 30 min ensured the homogeneity of the powders.

2.5 Bottling

The 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 in the bottle neck. Before closure of the vials, air was evacuated in a freeze-drier and replaced by argon. The vials were closed inside the freeze-drier with the help of a hydraulic device and then sealed with aluminium caps to prevent accidental opening during storage and transport. Colour-coded caps were used for easy identification of the different GM levels: nominal 0 g/kg = silver, nominal 5 g/kg = blue, nominal 10 g/kg = red, nominal 100 g/kg = brown, consistent with the cap colours of previous IRMM CRMs. Each of the vials was identified by a numbered label indicating the GM concentration level (Figure 1). Following the inventorying and the selection of vials for future analysis according to a random stratified sampling scheme, the bottles were brought to a storage room for long-term storage in the dark at 4 °C.

Figure 1: Prototype labels for the ERM-BF426 series. The denotation "blank" was used for the 0 g/kg 305423 soya powder, while "level 1", "level 2" and "level 3" refer to the nominal 5 g/kg, 10 g/kg and 100 g/kg 305423 soya, respectively.

2.6 Processing control

The residual mass fraction of water was determined by volumetric KFT in randomly selected bottles from each of the powder mixtures (Table 2). As a result of the drying steps during the processing, the water mass fractions in the final CRM powders were quite low (below 20 g/kg).

Analysis of the hygroscopic behaviour of ERM-BF426c samples by volumetric KFT showed a water uptake of 15.2 g/kg and 36.0 g/kg following incubation during 1 h at 43 % and 75 % relative humidity, respectively. Similar results can be expected for the other CRMs. As a result of the strong hygroscopicity of the powders, it is recommended to close the vials immediately after taking a sample.

Table 2: Water mass fraction in ERM-BF426 CRMs determined by volumetric KFT (*N* = 10, *n* = 1)

CRM	Water mass fraction [g/kg]	
	$\bar{x}$	*S*
ERM-BF426a	4.7	0.7
ERM-BF426b	15.7	2.5
ERM-BF426c	6.7	1.5
ERM-BF426d	7.9	1.1

Five randomly selected bottles from each of the powder mixtures were analysed for their particle size distribution based on laser diffraction patterns (PSA, Sympatec, Clausthal-Zellerfeld, DE). From each bottle, 2 subsamples were analysed. Note that the data generated by this analysis are based on the equivalent volume diameter fraction (see legend of Figure 2). The powders had very similar particle size distribution profiles (Figure 2), with a median particle size of 100 ± 22 μm ($\bar{x}$ ± *s;* based on the data from all four CRMs) and a maximum particle size below 730 µm. The average particle size, calculated by the PSA software, was 115 µm, 134 µm, 136 µm and 149 µm for ERM-BF426a, b, c and d respectively ($\bar{x}$ ± *s* for all powders = 134 ± 14 µm). Visual inspection of the powders, however, showed that some coarser particles are present in the bottles, which may result from particle aggregation or from insufficient milling. Their rare occurrence should, however, not affect the certified value or the use of the CRMs. The average particle size of 134 μm was used for the calculation of the minimum sample intake for the four CRMs (Section 3.2).

Maximum and average particle sizes were also confirmed by sieve analysis using ten sieves with meshes ranging from 45 µm to 2000 µm (Figure 2). Each CRM was analysed once (*N* = 1), using the combined contents of 10 bottles to reach the recommended sample intake of 10 g. The maximum particle size was below 710, 355, 500 and 500 µm for ERM-BF426a,b,c,d, respectively, and the median of the distribution for ERM-BF426a,b,c was 150 µm and for ERM-BF426d 90 µm. It is concluded from the results of both particle size analysis methods that the powders are sufficiently fine for an adequate extraction of genomic DNA [5].

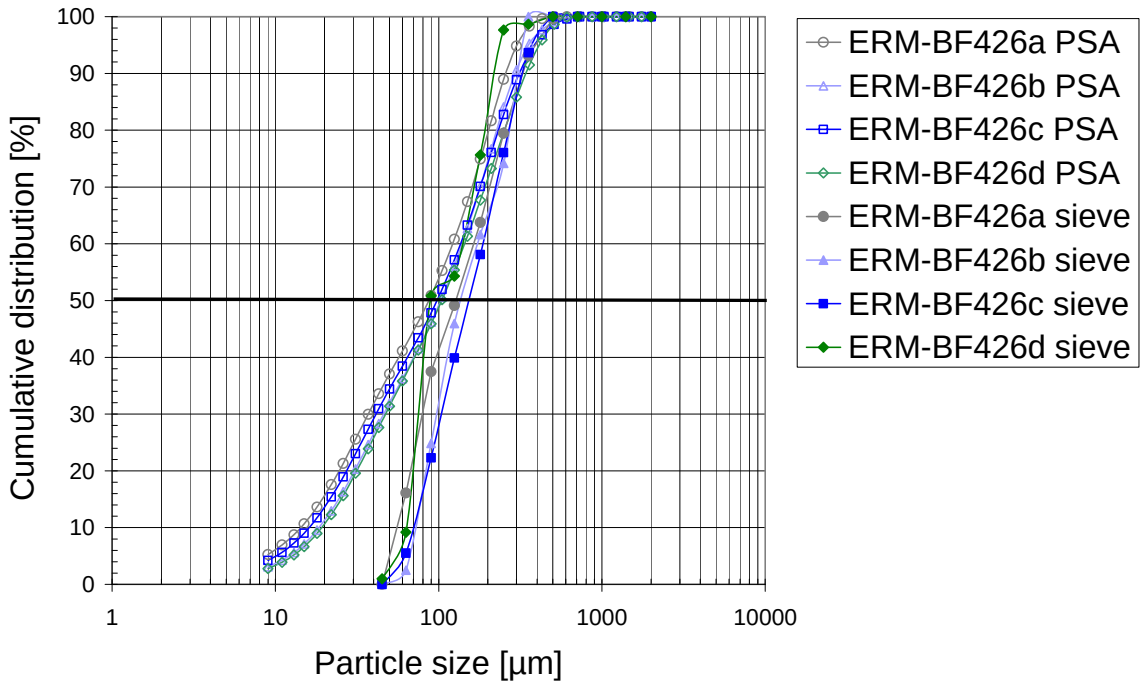

Figure 2: Average particle size distribution in ERM-BF426 by PSA (*N* = 5, *n* = 2) and sieving analysis (*N* = 1). The sieving data are cumulative mass fractions, but the cumulative distribution of particles derived from laser light scattering data (PSA) is based on their equivalent volume diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles.

Three of the described CRMs are mixtures of GM and non-GM soya powders, produced gravimetrically and certified for their GM powder mass fraction. Quantification of the GM content is, however, based on rt-PCR, which measures DNA copy number ratios. The mass fraction of DNA in both pure base materials was investigated in order to verify if the GM DNA copy number ratio was conserved in the gravimetric powder mixtures. The DNA mass was determined by a slight modification of the classical fractionation method developed by Ogur and Rosen [6]. Following the sequential removal of ethanol-, ethanol-ether- and acid-soluble compounds, the DNA was obtained by repeated acidic extraction with 0.84 mol/L perchloric acid at 70 °C. The mass of DNA was measured spectrophotometrically after derivatisation with diphenylamine, which reacts specifically with 2-deoxyriboses linked to purine nucleobases [6, 7]. The results are shown in Table 3. To reveal a difference between both powders, the ratio of their extractable DNA mass fractions was calculated as follows:

$$\frac{\text{Extractable mass of DNA in 100 mg 305423 soya powder}}{\text{Extractable mass of DNA in 100 mg non - GM soya powder}}$$

Table 3: Comparison of the DNA mass fraction extracted from GM and non-GM soya powders

DNA extraction method	*N*	Average ratio GM/non-GM	*U* (*k* = 2)
Modified Ogur & Rosen [6]	9	1.06	0.15

A *t*-test confirmed that there was no significant difference between the DNA mass fractions in the powders (95 % confidence level). Although this may suggest that the certified GM powder mass fractions equal corresponding GM copy number fractions, the customer is reminded that IRMM currently only certifies these materials for their GM powder mass fraction.

The mass fraction of 305423 soya in all four CRMs was analysed by rt-PCR targeting the specific T-DNA insertion in this soya in order to verify the consistency of the certified mass fractions in these CRMs. Genomic DNA was extracted from 200 mg powder samples using the GeneSpin genomic DNA extraction method (Eurofins GeneScan GmbH, Freiburg, DE). Real-time PCR measures copy numbers of the targeted DNA sequences but is calibrated with mass fractions of pure 305423 soya powder. Genomic DNA extracted from the 100 % GM powder is diluted in water and analysed by rt-PCR, producing a calibration curve ranging from 0.1 to 25 % mass fraction GM soya. The absolute GM mass fraction in a sample was furthermore related to the mass fraction of the soya-specific *le1* reference gene, calculated from a calibration curve of the same pure 305423 soya DNA diluted in water (mass fraction interval 1 to 100 %). The GM quantity is therefore expressed as a relative mass fraction. The efficiency of the amplification was determined from the slope of the regression line between the calibrant's mass fractions and the obtained Ct-values; for all standard curves, the efficiency was within the limits of the rt-PCR control chart. The limit of detection (LOD) was calculated as $(3.3*s)/b$, with s representing the standard deviation for the results of a triplicate reaction on the lowest GM mass fraction analysed and b the slope of the calibration curve. The results of the GM quantification for the four CRMs are shown in Table 4. Quantification of the GM mass fraction ratio in the powders by rt-PCR confirmed the consistency of the gravimetrically prepared mass fractions of ERM-BF426. However, one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements of unknown samples as DNA- and/or protein-based GM quantification may vary with the particular matrix and the soya variety tested [8].

Table 4: GM quantification in ERM-BF426 CRMs by event-specific real-time PCR

CRM	305423 soya copy number ratio [1] [g/kg]	*U* (*k* = 2) [g/kg]
ERM-BF426a	< 0.8 [2]	-
ERM-BF426b	4.8 [3]	0.9
ERM-BF426c	9.1 [3]	1.6
ERM-BF426d	87.8 [3]	12.2

[1] Real-time PCR measures the copy number ratio of the targeted GM DNA sequence in relation to the reference gene, calibrated with DNA extracted from pure GM powder and diluted in water.
[2] The measured value was below the LOD of the method (0.8 g/kg) for all three subsamples from five random bottles (N = 5, n = 3) of which each was measured in three replicates.
[3] Average of the rt-PCR results obtained by measuring five subsamples from each of five random bottles (N = 5, n = 5), with each subsample measured in three replicates.

3 Homogeneity

In order to ensure that the CRMs are sufficiently homogeneous, 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 before using soya materials processed in the same way as described for the 305423 soya [9]. Here we only report on the results of a homogeneity study performed on each of the three GM soya mixtures. Additionally, the recommended minimum sample intake is discussed.

3.1 Homogeneity study

The homogeneity of ERM-BF426 with respect to the event 305423 soya mass fraction was investigated by rt-PCR using bottles selected according to a random stratified procedure. The homogeneity of one of the CRMs, ERM-BF426d, was investigated using 15 bottles that were analysed in random order using a sample intake of 200 mg powder. As ERM-BF426b and c were processed and mixed in the same way as ERM-BF426d, their 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 bottle 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 [10]:

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

(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 were converted into relative uncertainties and were expressed in percentage (Table 5). The larger of both values was included into the calculation of the overall uncertainty on the certified values (Section 6.2).

Table 5: Standard uncertainties due to heterogeneity between bottles of dry-mixed 305423 soya CRMs, as analysed by rt-PCR

CRM	Number of samples analysed	Relative between bottle heterogeneity ($s_{bb,rel}$) [%]	Relative maximum hidden heterogeneity ($u^*_{bb,rel}$) [%]
ERM-BF426b	N = 5, n = 5	- [1)]	4.8
ERM-BF426c	N = 5, n = 5	3.1	3.6
ERM-BF426d	N = 15, n = 5	2.5	2.1

[1)] As MS_{bb} was smaller than MS_{wb}, $s_{bb,rel}$ 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 GeneSpin method. The assumption that a subsample of this size is representative for the whole batch was investigated as follows.

The mass density of the non-GM soya seed powder was determined by so-called tap-density measurements using the procedure described in [11]. Taking into account the mass density determined for the non-GM soya (0.64 g/mL) and the particle size distribution (average particle size of approximately 134 µm as equivalent volume diameter), it was calculated that a 200 mg sample roughly contains 2.5×10^5 powder particles. Consequently, 200 mg of ERM-BF426b (nominal 5 g/kg) would still contain $> 10^3$ 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

The short-term stability of dried soya seed powder (ERM-BF425) was investigated by analysis of DNA integrity and GM mass fraction ratio following isochronous incubation of bottles at 60 °C for up to 8 weeks [12]. It was concluded that dried soya CRMs could be shipped under ambient conditions without negative effects on their stability.

Because of the different oil composition of the 305423 soya compared to the 356043 soya mentioned above, we re-investigated the short-term stability of this CRM using an isochronous approach [13]. ERM-BF426d was chosen for this study as it contains the highest GM mass fraction of the four soya CRMs (nominal 100 g/kg). Five bottles were incubated at either 4 °C, 18 °C or 60 °C during 2 and 4 weeks, and several subsamples from each bottle were analysed for stability of the DNA in the matrix. A similar number of reference samples was likewise analysed following incubation at a reference temperature of -70 °C during the 4 weeks. Genomic DNA was extracted from the samples by the GeneSpin method and visualised by gel electrophoresis. No substantial DNA degradation was seen in any of the samples. Each DNA extract was also analysed by event-specific rt-PCR to reveal changes in GM quantification (Figure 3). Scrutinising the data obtained, the Grubbs tests revealed a single outlier (95 % confidence level) among the samples exposed for 4 weeks to 4 °C, but it was retained as no technical reason to exclude it was found. Regression analysis revealed a trend over the time period of 4 weeks (*t*-test, 95 % confidence level) for the samples incubated at 4 °C and 18 °C, but not for those exposed to 60 °C. As the trend was towards higher values in relation to the incubation time, and as it was absent at 60 °C, the data are presumably no reflection of DNA degradation.

It was concluded from this and the previous study [12] that the uncertainty due to degradation during dispatch is negligible for all four CRMs. ERM-BF426 can be shipped under ambient conditions.

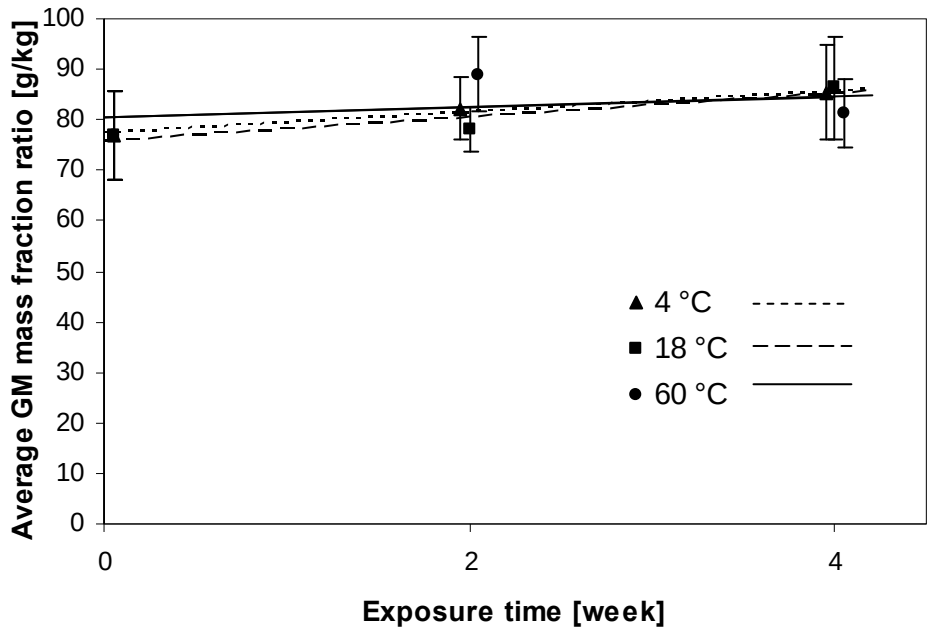

Figure 3: Short-term stability of ERM-BF426d at 4 °C (dotted regression line), 18 °C (dashed line) and 60 °C (solid line) as analysed by rt-PCR following the incubation of bottles for 2 or 4 weeks at the temperatures indicated or at the reference temperature of -70 °C (0 weeks exposure time). The bars indicate the interval $\bar{x} \pm s$ for $N = 5$ and $n = 5$ (-70 °C and 4 °C) or $n = 3$ (18 °C and 60 °C).

4.2 Long-term stability

The stability of the 305423 soya powder was unaffected by short-term incubation at elevated temperatures (Section 4.1), similarly to what was observed for the other soya matrix [12]. The 305423 soya oil, moreover, has an increased stability against oxidation compared to non-GMO soya oil. There is, therefore, no reason to think that the 305423 soya CRM would be less stable than other soya CRMs during long-term storage under controlled conditions (i.e. at 4 °C in the dark). Therefore, it was decided to rely on IRMM's stability monitoring experience with soya powder CRMs.

From post-certification stability analysis, the long-term stability of soya CRMs (i.e. ERM-BF410) during storage has been monitored at IRMM for a total of almost 6 years, using ELISA and/or event-specific rt-PCR methods (Figure 4, based on own unpublished results). Although visually there seems to be a downward trend in the stability data over the time period investigated, statistical evaluation revealed this to be insignificant (p = 0.15, t-test, 95 % confidence level). The relative standard uncertainty of the long-term stability (u_{lts}) [14], calculated from the available stability data, was approximately 2.2 % of the certified value per year.

It can be concluded that the storage conditions at IRMM are suited for the long-term storage of soya CRMs. As for all GMO CRMs, a post-certification monitoring scheme is put in place in order to continue monitoring the stability of ERM-BF426.

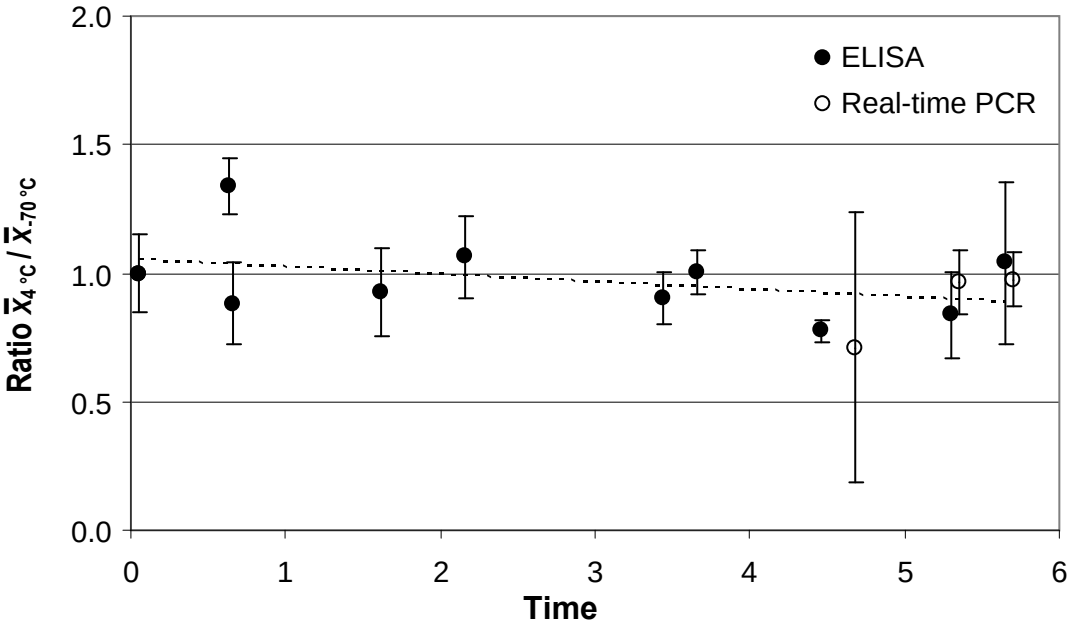

Figure 4: Long-term stability of dried soya seed powder (ERM-BF410, Roundup Ready soya) stored at 4 °C for various time periods, based on ELISA (●) and rt-PCR (○) measurements. The stability is expressed as the ratio between the GM mass fraction ratio in samples stored at 4 °C and that in samples stored 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 Characterisation

The four CRMs under the label ERM-BF426 are soya powder CRMs processed from pure non-GM and pure GM base materials. While ERM-BF426a is prepared from the pure non-GM blank material, ERM-BF426b,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 305423 event. If we assume that the purity of the non-GM powder is 100 %, which was supported by the data (Section 2.1), the GM mass fractions can be calculated according to the following formulas:

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

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

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

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

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).

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

Table 6: Characterisation of ERM-BF426 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]	
BF426d	1000	7.7	399.96	1000	7.8	3600.04	100
BF426c	100.0 [1)]	7.2	399.78	1000	7.8	3600.22	10.0
BF426b	10.0 [2)]	8.8	2001.01	1000	7.8	1998.99	5.0
BF426a	n.a.	n.a.	0	1000	7.8	4000.00	< 0.8 [3)]

[1)] For the preparation of BF426c, the nominal 100 g/kg GM soya was used.
[2)] For the preparation of BF426b, the nominal 10 g/kg GM soya was used.
[3)] Based on the LOD of the method.

6 Certified values and uncertainty budgets

6.1 Certified value

The certified value is based on the masses of dried powder of GM seeds and non-genetically modified seeds used in the gravimetrical preparation. The masses of the powders are corrected for their respective water mass fractions and their estimated 305423 soya purity (see Table 6 in Section 5).

The seed batches used for the processing of these powders were thoroughly checked for any impurity. No indication of the presence of the 305423 soya was found in the non-GM seed lot by rt-PCR, confirming the quality control results of the company supplying the seeds (Section 2.1). Processing controls additionally confirmed that the powder used for the production of ERM-BF426a did not contain traces of the 305423 soya above the LOD of the applied rt-PCR method (Table 4).

For the GM seeds, no indication was found for the absence of the 305423 soya in any of the individual seedlings raised from the GM seed lot when measured by event-specific rt-PCR; quality control on a larger number of individual seeds by the company supplying the soybeans revealed that all seeds carried the transgene (Section 2.1). Processing controls confirmed that ERM-BF426b,c,d contained the expected GM copy number ratio (Table 4).

As a consequence of the absolute purity of the seed batches used, the CRMs based on gravimetrical mixtures of GMO and non-GMO powders could be certified for values identical to the intended nominal GM mass fractions (Table 7).

6.2 Uncertainty budget

Controlled processing techniques in combination with purity controls of the GM and non-GM seeds and the derived powder base materials allowed certifying the GM mass fractions in the CRMs with rather small uncertainties.

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 [15].

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 (< 0.8 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 0.3 g/kg, which is below the certified < 0.8 g/kg.

Table 7: Uncertainty budgets for the mass fractions of 305423 soya in ERM-BF426

ERM	Certified value [g/kg]	Standard uncertainty contribution [g/kg]						Expanded uncertainty U (k = 2) [g/kg]
		u_{bb} 2)	u_{lts} 3)	$u_{char,1}$ 4)	$u_{char,2}$ 5)	$u_{char,3}$ 6)	$u_{char,4}$ 7)	
BF426a	**< 0.8** [1)]	n.a.	n.a.	n.a.	n.a.	0.2338	n.a.	**-**
BF426b	**5.0**	0.2416	0.1123	0.0075	0.0001	0.2338	0.0540	**0.8**
BF426c	**10.0**	0.3579	0.2245	0.0130	0.0001	0.2338	0.1081	**1.0**
BF426d	**100**	2.4607	2.2451	0.0922	0.0009	0.2338	1.0807	**7**

1) With a 95 % probability, the certified value is below this level.
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 (0.7 g/kg) and the highest water mass fraction found in any of the powders used for mixing (8.8 g/kg for the 10 g/kg GM powder).
6) Standard uncertainty introduced by the purity of the non-GM base material (LOD = 0.8 g/kg), based on the half-width of the interval between 0 and 0.8 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).

7 Metrological traceability

The ERM-BF426 series is composed of four reference materials certified for the mass fraction of event 305423 soya seed powder. The certified values are based on gravimetric dry-mixing of non-modified soya seed powder with event 305423 soya seed powder.

The respective certified values are 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 values for the GM copy number ratio measured by rt-PCR could potentially differ from the certified GM mass fraction as a result of different DNA extraction efficiencies from GM and non-GM powders.

8 Intended use and instructions for use

ERM-BF426a,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.

References and acknowledgements

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Abstract

This report describes the processing and certification of four soya seed powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) 305423 soya (ERM-BF426a,b,c,d). The materials were processed and certified in 2007 by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), according to the principles of ISO Guide 34.

Homozygous seeds of GM 305423 soya and of a comparable non-GM soya line were dried and ground to GM and non-GM base powders. A non-GM pure material and three gravimetric mixtures of non-GM and GM soya powder (containing respectively 5.0, 10.0 and 100 g/kg GM soya) were prepared by dry-mixing. The remaining > 500 µm particles were then sieved out from each of these powders, milled a second time, and added back to the main fraction. The certified values of these CRMs were calculated from the gravimetric preparations, taking into account the GM purity of the base materials and their water mass fraction. The certified values were confirmed by event-specific real-time PCR as independent verification method (measurements within the scope of accreditation to ISO/IEC 17025).

The four CRMs belonging to the ERM-BF426 set were certified to contain the following 305423 soya mass fractions:

CRM	**Certified value: 305423 soya mass fraction [1] [g/kg]**	**Uncertainty [2] [g/kg]**
ERM-BF426a	< 0.8 [3]	-
ERM-BF426b	5.0	0.8
ERM-BF426c	10.0	1.0
ERM-BF426d	100	7

[1] The certified value is based on the mass fraction of 305423 soya seed powder mixed in non-genetically modified soya seed powder and taking into account their respective 305423 soya purity and their water mass fraction. The certified value is traceable to the SI.
[2] The certified uncertainty is the expanded uncertainty (*U*) estimated in accordance with the Guide to the Expression of Uncertainty in Measurement with a coverage factor $k = 2$, corresponding to a level of confidence of about 95 %.
[3] With a 95 % probability, the value of the material is below this level.

The CRMs are intended to be used for quality control or calibration of methods for the quantification of the 305423 soya mass fraction in food and feed. The CRMs are available in glass bottles containing 1 g of dried soya powder closed under argon atmosphere. The minimum amount of sample to be used per analysis is 200 mg.

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

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