Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 59122 Maize

Certified Reference Materials ERM®-BF424 (ERM®-BF424a, ERM®-BF424b, ERM®-BF424c, ERM®-BF424d)

Report EUR 22333 EN

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

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

European Commission
Directorate-General Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
CRM Sales
European Commission
Directorate-General 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.irmm.jrc.be
http://www.jrc.ec.europa.eu
http://www.erm-crm.org

Legal Notice
Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use which might be made of the following information.

EUR Report 22333 EN
ISBN 92-79-02755-7
ISSN 1018-5593
Luxembourg: Office for Official Publications of the European Communities

© European Communities, 2006

Reproduction is authorised provided the source is acknowledged
Printed in Belgium

CERTIFICATION REPORT

The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 59122 Maize

Certified Reference Materials ERM®-BF424 (ERM®-BF424a, ERM®-BF424b, ERM®-BF424c, ERM®-BF424d)

D. Gancberg, S. Trapmann, S. Broeders, K. Teipel, P. Conneely, M. Contreras, P. Corbisier, E. Hannes, S. Gioria, M. Van Nyen, H. Schimmel, H. Emons

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

Report EUR 22333 EN

ADDENDUM

Certification report ERM-BF424

EUR 22333 EN

Additional information

ANNEX
Use of ERM-BF424 for measurement results based on copy number ratios

For the preparation of the CRMs ERM-BF424, Pioneer Hi-Bred International (IA, USA) supplied seeds of non-modified maize (variety 'X1083A') and GM 59122 maize to the IRMM. According to Pioneer the GM 59122 maize seeds (unique identifier DAS-59122-7) are hybrid seeds, where the GM donor of the GM event is the male parent.

ERM-BF424 has been certified for its GM mass fraction and not for its GM copy number ratio. If users intend to use ERM-BF424 for GM measurement results expressed in copy number ratios, the estimation of the copy number ratio and the related uncertainty needs to be carried out according to the principles explained in ERM Application note 4 [2].

For the estimation of the copy number ratio the measurement unit of the certified value given in g/kg to the measurement unit '1' for ratios, expressed in percent, is required. Furthermore, the hybrid status of the seeds used for the production of the CRMs needs to be considered as well as the DNA extractability of the non GM and GM seed powder.
This estimation is linked to additional standard uncertainties, which need to be taken into consideration in the combined expanded uncertainty. These additional standard uncertainties refer to:
(1) Measurement uncertainty of the quantification method
(2) Possible variation of the maize genome size [3]
(3) Possible effect caused by endoreduplication
(4) Possible effect caused by the GM trait introduction of the maize event.
As the GM trait introduction via the male parent could not be verified by IRMM it is strongly recommended to take both possibilities of the GM trait introduction (introduction via the female or male parent) into consideration when calculating the combined expanded uncertainty. On the other hand it is reasonable to consider the maize seeds used for the production of the matrix to be hybrids into which the GM trait has been introduced by one parent.
The principles of the approach to estimate the copy number ratio value and its expanded combined uncertainty of a mass fraction certified maize CRM are outlined in ERM Application note 4 [2].

References
[1] Trapmann S, Conneely P, Contreras M, Corbisier P, Gancberg D, Hannes E, Gioria S, Muñoz-Pineiro A,Van Nyen M, Schimmel H, Szilágyi S, Emons E (2005) The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 1507 Maize - Certified Reference Materials ERM®-BF418, EC certification report EUR 21689 EN, ISBN 92-894-9748-3
[2] Application note 4: European Reference Materials - Use of certified reference materials for the quantification of GMO in food and feed.
http://www.erm-crm.org/html/ERM_products/application_notes/application_note_4/index.htm
[3] Poggio L, Rosato M, Chiavarino AM, Naranjo CA 1998: Genome Size and Environmental Correlations in Maize (Zea mays ssp. mays, Poaceae), Annals of Botany 82, 107-115

Geel, February 2010

SUMMARY

This report describes the preparation and certification of maize powder CRMs with different mass fractions of genetically modified (GM) 59122 maize powder (Certified Reference Materials ERM®-BF424a, ERM®-BF424b, ERM®-BF424c and ERM®-BF424d). The materials were processed and certified in 2006 by the European Commission, Directorate General Joint Research Centre, the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium. Seeds of non-modified maize and 59122 maize both supplied by Pioneer Hi-Bred International (Johnston, IA, USA) were rinsed with demineralised water, drained and dried at 30 °C in order to minimise dust contamination from other crops. After a two step grinding process, transforming the seeds into a non-modified maize powder and a 59122 GM maize 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. The 59122 mass fractions of ERM®-BF424 were verified with the help of DNA-based detection methods. The CRMs are available in glass bottles containing 1 g of maize powder packed under argon atmosphere. The four CRMs of ERM®-BF424 were certified to contain the following 59122 mass fractions:

CRM	Certified value 59122 mass fraction [1] [g/kg]	Uncertainty [2] [g/kg]	
		Relevant below the certified value	Relevant above the certified value
ERM®-BF424a	< 1.2	n.a.	n.a.
ERM®-BF424b	1.0	0.2	1.2
ERM®-BF424c	9.9	0.8	1.4
ERM®-BF424d	98.7	5.8	5.9

[1] The certified value is based on the mass fraction of dried non-genetically modified powder and dried genetically modified powder mixed and corrected for the water content of the two materials before mixing. The certified value is traceable to the SI.

[2] The certified uncertainty is the expanded uncertainty estimated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) with a coverage factor k = 2, corresponding to a level of confidence of about 95 %. The expanded uncertainty for ERM®-BF424b covers the interval from 0.8 to 2.2 g/kg, for ERM®-BF424c covers the interval from 9.1 to 11.3 g/kg and for ERM®-BF424d covers the interval from 92.9 to 104.6 g/kg.

The minimum amount of sample to be used is 100 mg.

Table of contents

Glossary

ANOVA	Analysis of Variance
b	slope in equation of linear regression $y = a + bx$
CRM	Certified Reference Material
CRL	Community Reference Laboratory
Cry34Ab1	insecticidal crystal protein that helps to control corn rootworm larvae pest
Cry35Ab1	insecticidal crystal protein that helps to control corn rootworm larvae pest
CTAB	cetyltrimethylammonium bromide
Ct-value	number of PCR cycles to pass a set threshold
CV	coefficient of variation
DNA	deoxyribonucleic acid
ERM®	trademark European Reference Materials
ELISA	Enzyme-Linked Immuno Sorbent Assay
FAM	6-FAM™ fluorescent dye
gDNA	genomic DNA
GM	genetically modified
GMO	genetically modified organism
hmg	high mobility group gene from *Zea mays*
IRMM	Institute for Reference Materials and Measurements
JRC	Joint Research Centre
k	coverage factor
KFT	Karl Fischer titration
LOD	limit of detection
LOQ	limit of quantification
N	number of samples analysed
n	number of subsamples analysed
n.a.	not applicable
NAA	neutron activation analysis
pat	phosphinothricin acetyltransferase
PCR	polymerase chain reaction
PSA	particle size analysis by laser diffraction
R^2	coefficient of correlation
rt-PCR	real-time PCR
s	standard deviation
SI	International System of Units
U	expanded uncertainty
u	standard uncertainty
u^*_{bb}	uncertainty component due to the inhomogeneity that can be hidden by method repeatability
UV	ultra-violet
$\bar{x}$	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), provided the GMO has been placed on the market in accordance with Community legislation [1]. This necessitates on the one hand to develop and validate reliable quantitative detection methods and on the other hand to develop and produce reference materials to calibrate and control the correct application of such detection methods. Therefore, mixtures of genetically modified (GM) and non-GM powders have been prepared and made available as Certified Reference Materials (CRMs).
According to European Commission regulation (EC) No 65/2004 [2] the event 59122 maize corresponds to the unique identifier DAS-59122-7. DAS stands for Dow AgroSciences, who claimed that the maize is genetically engineered to express three new proteins, Cry34Ab1, Cry35Ab1, and phosphinothricin acetyltransferase (pat). Cry34Ab1 and Cry35Ab1 are insecticidal crystal proteins discovered in Bacillus thuringiensis cultures that confer resistance to Coleopteran insects, pat confers tolerance to chemically synthesized phosphinothricin products such as glufosinate-ammonium and is used as a selectable marker.

This report describes the processing and certification of four CRMs of maize powder with different mass fractions of dried GM maize powder of the transformation event 59122
(< 1.2, 1.0, 9.9, 98.7 g/kg) by IRMM.

ERM®-BF424 has been produced by means of dry-mixing techniques in order to minimise DNA and protein degradation during the processing.

2 CRM preparation

2.1 Characterisation of the base materials

For the preparation of the CRMs, Pioneer Hi-Bred International (IA, USA) supplied seeds of non-modified maize and GM 59122 maize to IRMM. Fifty kilogram of non-modified maize and 25 kg of 59122 maize were used for the processing of ERM®-BF424. The GM seeds are hybrid seeds and heterozygous for the transgene (variety X4K504Z). The non-modified seeds are from the hybrid variety X1083A.
Pioneer Hi-Bred International carried out quality controls to assess the purity of the 59122 seed batch by testing 200 randomly chosen seeds for the presence of the Cry34Ab1 protein using an Enzyme-Linked Immuno Sorbent Assay (ELISA) specifically designed for the Cry34Ab1 protein. All 200 seeds tested positive for the Cry34Ab1 protein. The purity of the non-GM seed batch was determined using an event-specific PCR detection method for 59122 maize [3]. According to the supplier, four pooled samples of 75 seeds each were collected from the non-GM seed batch and subdivided in five flour subsamples each. All subsamples tested negative for the presence of the 59122 maize. From these data, the purity of the non-GM seed batch was estimated at IRMM to be 99 % free of 59122 maize at a 95 % confidence level.

The purity and genetic composition of these batches were verified at IRMM by analysing the genomic DNA (gDNA) extracted from leaves of seedlings. Seeds of the non-GM and GM batches (N = 60 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 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 average mass of DNA extracted $\bar{x} \pm s$ per mass of wet tissue was 109 ± 22 mg/kg for the GM tissue and 80 ± 16 mg/kg for the non-GM tissue.
Detection by real-time polymerase chain reaction (rt-PCR) was performed at IRMM following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA). Primer pairs specific for the event 59122 and the *hmg* endogenous maize gene have been used together with TaqMan® probes labelled with FAM. The cycle threshold values (Ct-value) determined for the 60 GM plants were compared to a calibration curve obtained from dilution series of gDNA extracted from pure 59122 powder. All GM plants tested positive for 59122 and the measured mass fraction average $\bar{x} \pm s$ of GM 59122 maize, estimated via the transgenic copy number measured relative to the copy number of an endogenous gene, was 1265 ± 81 g/kg (N = 60)[1)]. It was concluded that all 60 plants were heterozygous for the GM event. All non-GM plants were tested in the same way and found negative for the event 59122 (Table 1).

[1)] Due to the calibration with powders produced from seeds (and the genetic composition of the various tissue types), the rt-PCR results obtained for the gDNA extracted from plants can deviate considerably from pure GM powder.

Table 1: Purity test and genetic composition of the GM and non-GM seed batches used for the production of ERM®-BF424

Batch	PCR method performed and primers used [1)]	Number of plants tested	Number of 59122 positives	Number of 59122 negatives
Non-GM	Event-specific real-time PCR	60	0	60
GM	Event-specific real-time PCR	60	60	0

[1)] Primer sequences of the event-specific 59122 method have been provided by Pioneer Hi-Bred International under confidentiality agreement.

Additionally the purity of the ground non-GM base material was tested at IRMM. The analysis of randomly selected seeds and subsequent analysis of the powder (three DNA extractions from 100 mg powder each) indicated that no GM contamination was detected in the non-GM lot, i.e. the values obtained were all below the detection limit (LOD) of the rt-PCR method applied (Table 2).
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 59122 powder in nuclease free water and were found to be 1.0 g/kg and 2.9 g/kg, respectively.

Table 2: Quantification of GM 59122 contamination in the non-GM base material by event-specific rt-PCR using 100 mg samples for DNA extraction and 50 ng DNA per PCR reaction

Non-GM base material	***N*** [2)]	Mass fraction GM contamination [3)] [g/kg]
Event-specific rt-PCR [1)]	3	< 1.0

[1)] Primer sequences of the event-specific 59122 method have been provided by Pioneer Hi-Bred International under confidentiality agreement.
[2)] The DNA extract of each sample has been subjected to a rt-PCR analysis in triplicate.
[3)] The measured mass fraction is below the calculated LOD of 1.0 g/kg.

To verify the DNA mass fraction in both powders, a slight modification of the classical fractionation method developed initially by Ogur & Rosen was employed [5]. Following 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 [5, 6]. The ratio between the extractable DNA mass of the two materials was calculated as:

$$\frac{\text{Extractable mass of DNA in 100 mg 59122 maize powder}}{\text{Extractable mass of DNA in 100 mg nonGM maize powder}}$$

A DNA ratio of 1.00 ± 0.12 was found (Table 3), and a *t*-test demonstrated no significant difference at 95 % confidence level between the extractable mass of DNA of the two maize powders.

Table 3: Ratio of the precipitating DNA of GM and non-GM ground base material

Extraction method	*N*	Mass fraction ratio $\bar{x} \pm U\ (k = 2)$
Modified Ogur & Rosen [5]	9	1.00 ± 0.12

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 30 g/kg in the case of the non-GM seeds and 40 g/kg in the case of the GM seeds. 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 90 g/kg. For the second grinding step a sieve insert was used with 0.5 mm mesh width. Slow feeding of the mill ensured that the whole base material passed the sieve and that no selective grinding occurred. Care was taken to avoid that the material was exposed to temperatures above 40 °C during mixing.
Each ground base material was mixed in a turbula mixer for 30-45 minutes to improve equal distribution of the different parts of the maize kernels separated by the milling process. After measurement of the remaining water content it was decided to carry out an additional vacuum drying at 30 °C. 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 20 g/kg for the non-GM powder and 15 g/kg for the GM powder.

2.3 Gravimetric preparation of GM mixtures

The twice-ground base materials were used to produce powder mixtures containing mass fractions of 59122 maize powder at nominal levels of 0, 1, 10 and 100 g/kg. Prior to the dry-mixing, the 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 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 maize 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.

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 4).

Table 4: Water mass fraction of the four CRMs (*N* = 10, *n* = 1)

CRM	Water mass fraction [g/kg]	
	$\bar{X}$	*s*
ERM®-BF424a	11.3	2.9
ERM®-BF424b	19.2	4.4
ERM®-BF424c	16.9	2.5
ERM®-BF424d	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, 3 subsamples were analysed. The powders had a maximum particle size below 735 μm (Table 5) and a median particle size around 111 μm, used for the calculation of the minimum sample intake (Section 3.2) and the calculation of the uncertainty budget (Section 5.3).

Table 5: Particle size distribution of the CRMs, determined by laser light diffraction (*N* = 5, *n* = 3)

CRM	Particle number fraction according to their size [%] $\bar{X} \pm s$					
	< 90 µm	< 125 µm	< 180 µm	< 255 µm	< 515 µm [1)]	< 735 µm [1)]
ERM®-BF424a	47.5 ± 6.2	56.6 ± 7.4	70.0 ± 9.8	84.5 ± 9.5	99.6 ± 1.0	100.0
ERM®-BF424b	41.8 ± 2.1	50.0 ± 2.4	62.1 ± 3.0	77.7 ± 3.9	99.9 ± 0.3	100.0
ERM®-BF424c	42.5 ± 2.8	50.7 ± 3.4	62.7 ± 4.7	78.0 ± 6.0	99.9 ± 0.5	100.0
ERM®-BF424d	49.6 ± 6.8	59.6 ± 8.7	74.3 ±11.1	88.9 ± 9.5	100.0	100.0

[1)] For ERM®-BF424a six of the 15 subsamples gave results between 96.5 and 99.5 % for the particle size class < 515 µm. For ERM®-BF424b two of the 15 subsamples gave results between 99.0 and 99.6 % for the particle size class < 515 µm. For ERM®-BF424c one of the 15 subsamples gave results of 98.1 % for the particle size class < 515 µm. For all four CRMs the subsamples gave 100.0 % for the particle size class < 735 µm.

Additionally, a sieving test was carried out following ISO 3310-1 [7] using sieves with meshes of 90, 125, 180, 250, 500 and 710 µm (Table 6) 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.

Table 6: Particle size distribution of the CRMs, determined by sieving test (*N* = 1) using a sample intake of 10 g

CRM	Particle number fraction according to their size [%]					
	< 90 µm	< 125 µm	< 180 µm	< 250 µm	< 500 µm	< 710 µm
ERM®-BF424a	15	38	50	68	100	100
ERM®-BF424b	11	37	49	66	100	100
ERM®-BF424c	8	35	49	66	100	100
ERM®-BF424d	8	35	48	66	99	100

2.6 Confirmation of 59122 maize 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 1.0 g/kg and 2.9 g/kg, respectively (for details see Section 2.1). The results obtained can be found in Table 7.

Table 7: Quantification by event-specific 59122 real-time PCR. DNA was extracted from 100 mg powder sample intakes using the CTAB method [8]. The measurements were calibrated with pure GM powder

CRM	59122 mass fraction determined by event-specific rt-PCR [2) 3)] [g/kg]	*U* (*k* = 2) [g/kg]
ERM®-BF424a	< 1.0 [1)]	n.a.
ERM®-BF424b	0.9	0.2
ERM®-BF424c	9.8	2.0
ERM®-BF424d	94.2	8.3

[1)] The measured value was below the LOD of the method of 1.0 g/kg.
[2)] For each CRM the content of five randomly selected bottles was analysed and three subsamples (*N* = 5, *n* = 3) 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 7) were compared to the certified values (Section 5.3, Table 10). Quantification of the GM mass fraction of three mixtures of 59122 powders by rt-PCR proved to be consistent with the gravimetrically prepared mass fractions of CRM ERM®-BF424. 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 maize variety tested [9].

3 Homogeneity

3.1 Homogeneity study

In order to ensure that the CRMs are sufficiently homogenous, two strategies were followed.
1) Prior to the production of ERM®-BF424 a study was carried out using other maize powders [10] produced in the same way as the maize powders used for the preparation of ERM®-BF424. In this homogeneity study for maize powders, Au-spiked non-GM maize powder was dry-mixed with non-spiked non-GM maize powder and the homogeneity evaluated on the distribution of Au.
All materials used in the homogeneity study were processed in the same way as described for the ground base materials in Section 2.2. A part of the maize powder was spiked with Au. Afterwards a 100 g/kg mass fraction was produced and further diluted twice to reach mass fractions of 10 and 1 g/kg Au-spiked in non-spiked maize. The Au mass fraction of each of the three mixtures was determined with neutron activation analysis (NAA). The results showed the homogeneity of the dry-mixed maize powder at a sample intake level of 50 mg (Table 8) and confirmed the adequacy of the dry-mixing technology for the preparation of maize mixtures with different GM mass fractions.

Table 8: Homogeneity study on dry-mixed maize powder; results of Au determination by neutron activation analysis (NAA) with a sample intake of 50 mg (*n* = 6) [10]

Material	Mixture parts		Results NAA	
	Au-spiked [g]	Non-spiked [g]	Au mass fraction [mg/kg]	CV [%]
Au-spiked maize	1000	0	1300	4
Non-spiked maize	0	1000	0.005	10
100 g/kg mixture	100	900	132	6
10 g/kg mixture	10	990	13	5
1 g/kg mixture	1	999	1.2	15

2) In addition to this study, the homogeneity of ERM®-BF424 with respect to the 59122 maize mass fractions was measured with rt-PCR and analysed using ANOVA (Table 9). In order to determine the between-bottle variation and the maximum hidden heterogeneity of CRM ERM®-BF424, two event-specific PCR measurements on five randomly selected bottles were carried out. For ERM®-BF424b (nominal 1 g/kg) and ERM®-BF424c (nominal 10 g/kg), a CV of 9.9 % and of 8.5 % were found, respectively. For ERM®-BF424d (nominal 100 g/kg) the CV was lower than the maximum hidden heterogeneity and it was concluded that the CV contribution from the homogeneity u^*_{bb} is smaller than 5.8 %. 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 10) was valid.

Table 9: Homogeneity data of dry-mixed maize powder analysed by ANOVA (N = 5, n = 3)

CRM	Relative between bottle homogeneity [%]	u^*_{bb} [%]
ERM®-BF424a	n.a.	n.a.
ERM®-BF424b	9.9	12.0
ERM®-BF424c	8.5	4.0
ERM®-BF424d	n.a. [1)]	5.8

[1)] Using the ANOVA calculations, the mean sum of squares between bottle was inferior to the mean sum of squares within bottle, and the relative between bottle homogeneity could not be calculated.

3.2 Minimum sample intake for analysis

Most DNA extraction methods from plant powders use 100 mg sample intakes and the hypothesis that this quantity would be suitable for analysis was tested.

The mass density of the non-GM maize powder was determined by so-called tap-density measurements, carried out using the non-GM powder similarly to the procedure described in [11]. Taking into account the mass density (0.84 g/mL) and the particle size distribution (average particle size of 111 µm), it was estimated that the number of particles in a 100 mg sample is larger than 160×10^3. Consequently 100 mg of ERM®-BF424b (nominal 1 g/kg) contain around 160 GM particles. On this basis uncertainties due to sample inhomogeneity were estimated (Section 5.3). Additionally, referring to the particle size distributions it is advised to use sample intakes not smaller than 100 mg.

4 Stability

4.1 Short-term stability

It can be assumed that dried maize powder produced and stored under the same conditions behaves similarly. In order to assess whether special care must be taken during transportation, a short-term stability of dried maize powder was investigated. Therefore, previous data obtained [10] were accepted for the short-term stability of ERM®-BF424. During that short-term stability study on another material an isochronous approach [12] was applied. Bottles closed under argon and containing a nominal GM mass fraction of 10 g/kg were exposed to 60 °C for 2 and 8 weeks. The DNA integrity of the samples was analysed by gel electrophoresis, the extractable DNA content was determined by UV spectrometry, and the relative concentration of the transgenic sequence was verified by rt-PCR. The results were compared to results obtained for samples stored at a reference temperature of -70 °C.

UV measurements and rt-PCR data confirmed that samples can be exposed to temperatures of 60 °C for 2-3 weeks. Moreover, no DNA degradation was observed on the gels for any of the samples. Dried maize powder can therefore be shipped under ambient conditions [10]. Figure 1 summarizes the UV and rt-PCR data obtained during the study of 8 weeks.

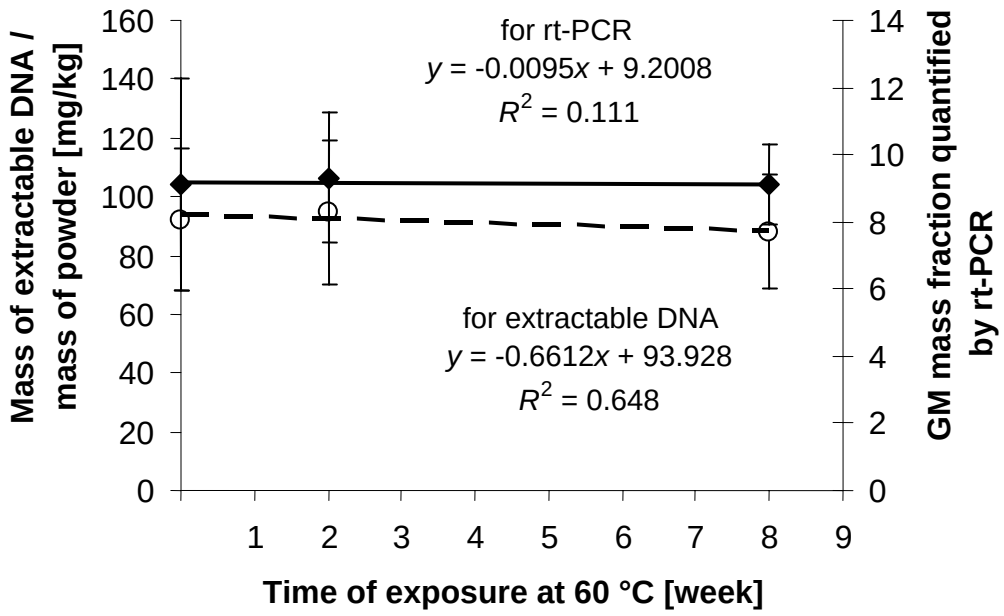

Figure 1: Short-term stability of ERM®-BF416 at 60 °C described by GM mass fraction quantified by rt-PCR (♦, regression line = full) or by mass of extractable DNA / mass of powder (O, regression line = dotted). The bars indicate the interval $\bar{x}$ ± *s* for *N* = 15 [10].

4.2 Long-term stability

Earlier productions of dry-mixed maize GM CRMs have been monitored by gel-electrophoresis, ELISA and rt-PCR for long-term stability during storage at 4 °C in the dark and under argon for at least 5 years (Figure 2). The monitoring of the CRMs did not indicate instability as the slope of the regression line was not significant (*t*-test, 95 % confidence interval). The uncertainty of the slope of the regression line (0.0230 g/kg per month) was used as the contribution due to instability of the CRMs upon storage (Table 10, Section 5.3). It can be assumed that the stability of maize powders dried to the same extent and stored under similar conditions is the same. Post-certification monitoring is being carried out at regular intervals in order to monitor the stability of ERM®-BF424.

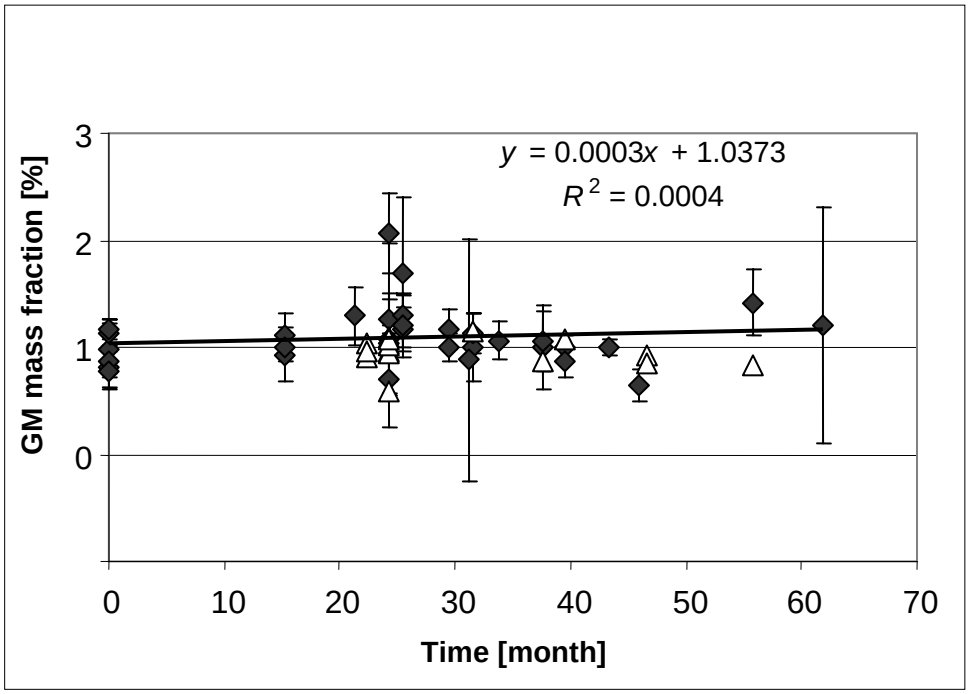

Figure 2: Long term-stability of maize CRMs stored at 4 °C for up to 62 months assessed by rt-PCR (♦) and ELISA (Δ), regression line = full. The bars indicate the interval $\bar{x}$ ± *s* for *N* = 3 to 15.

5 Certified mass fractions and uncertainty budgets

5.1 Metrological traceability

ERM®-BF424a, ERM®-BF424b, ERM®-BF424c and ERM®-BF424d are four reference materials certified for the mass fraction of 59122 maize powder. The certified mass fractions are based on gravimetric dry-mixing of non-modified maize powder with 59122 maize powder.
The certified value is traceable to the SI. The traceability chain to the kilogram is based on the use of calibrated balances, a thorough control of the weighing procedure and the control of the purity of the used seeds.
It must, however, be emphasised that the ratio between transgenic DNA and endogenous (target taxon specific) DNA in the reference materials may significantly deviate from the certified powder mass fraction values due to the genetic composition of the maize tissues. Additionally, the mass fraction DNA / dry powder in the GM and non-GM base material cannot be determined with high accuracy due to the relatively large uncertainty inherent to quantification of the DNA mass.
Furthermore, the user of the certified reference material should bear in mind that different efficiencies of the DNA extraction from GM and non-GM powders, if occurring, influence the GM mass fraction measured by rt-PCR.

5.2 Certified value

The certified value is based on the masses of dried genetically modified powder and dried non-genetically modified powder used in the gravimetrical preparation. The masses of the powders are corrected for their water content and their estimated 59122 purity. The GM mass fraction is calculated as:

$$\frac{\text{Corrected mass GM powder}}{(\text{corrected mass GM powder}) + (\text{corrected mass nonGM powder})}$$

For the purity of the GM base material the genetic identity of randomly selected kernels has been checked (see Section 2.1). No evidence could be found that non-GM seeds were among the GM seeds. 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 is higher than 97.5 % and the average between 100 % and 97.5 % was used for the calculation of the various mass fractions (59122 mass fraction purity = 98.8 %). The non-GM impurity was taken as 0 % as no contamination was found and it was concluded that the material more likely contains a GM mass fraction closer to 0 % than to half of the LOD of the applied quantification method (0.05 %).

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 10).
The combined uncertainty of the certified value comprises the uncertainties introduced due to the weighing procedure, the serial dilutions, the water mass fraction determination, the inhomogeneity at the recommended sample intake of 100 mg, the long-term stability of the material and the purity of non-GM and GM base material. The uncertainty contributions of the purity of the two base materials have only to be considered for the combined upper limit of the uncertainty. Even in case of a GM contamination in the non-GM material the GM mass fraction can only increase, but can never decrease. As no negative seeds were found during purity assessment it is more likely that the true value of the GM base material is

closer to 100 % than to 98.8 %. For the given reasons the uncertainty contribution of the purity values of the two base materials is not considered when calculating the lower limit of the expanded uncertainty. 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 stability testing has been estimated by monitoring various nominal 10 g/kg maize CRMs by ELISA or rtPCR over time (Figure 2). A coverage factor of 2 was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % (Table 10).

Table 10: Uncertainty budget for the mass fraction of 59122 maize in ERM®-BF424 in g/kg

CRM value		Standard uncertainty contributions						U relevant below the certified value (k = 2)	U relevant above the certified value (k = 2)
		u_1 [1)]	u_2 [2)]	u_3 [3)]	u_4 [4)]	u_5 [5)]	u_6 [6)]	$U_{1, 2, 3, 4}$	$U_{1, 2, 3, 4, 5, 6}$
ERM®-BF424a	< 1.2 [7)]	n.a.	n.a.	n.a.	n.a.	0.5774	n.a.	n.a.	n.a.
ERM®-BF424b	1.0	0.0029	0.0019	0.0771	0.0276	0.5774	0.0040	0.2	1.2
ERM®-BF424c	9.9	0.0235	0.0158	0.2437	0.2755	0.5774	0.0403	0.8	1.4
ERM®-BF424d	98.7	0.1664	0.1116	0.7707	2.7553	0.5774	0.4031	5.8	5.9

[1)] Uncertainty introduced by the mass determination (mainly based on the uncertainty of the balance).
[2)] Uncertainty introduced by the dilution technique. For ERM®-BF424b three dilution steps, for ERM®-BF424c two dilution steps and for ERM®-BF424d one dilution step were taken into consideration. (The relative standard uncertainty of the water content determination KFT method in maize was estimated to be 11.3 % for n = 3.)
[3)] Uncertainty introduced by the inhomogeneity at 100 mg level (average particle size of 111 µm, mass density of 0.84 g/mL).
[4)] Uncertainty introduced by the stability of the maize powders, estimated for 12 months.
[5)] Uncertainty introduced by the purity of non-GM base material.
[6)] Uncertainty introduced by the purity of GM base material.
[7)] The purity of the non-GM base material was estimated to be below the LOD of the method applied, and this LOD was used to calculate the certified property of the blank material according to the GUM [13].

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] 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.
[6] 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.
[7] ISO 3310-1: 2000. Test sieves – Technical requirements and testing. Part 1: Test sieves of metal wire cloth.
[8] Pietsch K., Waiblinger HU., Brodmann P., Wurz A. (1997): Screeningverfahren zur Identifizierung 'gentechnisch veränderter' pflanzlicher Lebensmittel, Deutsche Lebensmittel-Rundschau 93, 35-38.
[9] Website www.erm-crm.org, Application Note 4: Use of Certified Reference Materials for the quantification of GMO in food and feed.
[10] Trapmann S., Charoud-Got J., Conneely P., Contreras M., Corbisier P., Gancberg D., Hannes L., Gioria S., Muñoz-Pineiro A., Van Nyen G., Schimmel H., Szilagy S., Emons H. (2005): The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of MON 863 Maize, EC certification report EUR 21574, ISBN 92-894-9195-7.
[11] ISO/IEC 3953:1993 Metallic powders - Determination of tap density.
[12] 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.
[13] ISO (1995): Guide to the Expression of Uncertainty in Measurement, ISBN 9267-101889

Acknowledgements

The authors would like to thank G. Roebben and A. Lamberty (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 (EQUALIS AB, SE) for their critical comments.

European Commission

EUR 22333 EN – DG Joint Research Centre, Institute for Reference Materials and Measurements –
The Certification of Reference Materials of Dry-Mixed Maize Powder with different Mass Fractions of 59122 Maize, ERM®-BF424a, ERM®-BF424b, ERM®-BF424c, ERM®-BF424d
Authors: D. Gancberg, S. Trapmann, S. Broeders, K. Teipel, P. Conneely, M. Contreras, P. Corbisier, E. Hannes, S. Gioria, M. van Nyen, H. Schimmel, H. Emons
Luxembourg: Office for Official Publications of the European Communities
2006 – 18 pp. – 27.0 x 29.1 cm
EUR 22333 EN- Scientific and Technical Research series; ISSN 1018-5593

Abstract

This report describes the preparation and certification of maize powder CRMs with different mass fractions of genetically modified (GM) 59122 maize powder (Certified Reference Materials ERM®-BF424a, ERM®-BF424b, ERM®-BF424c and ERM®-BF424d). The materials were processed and certified in 2006 by the European Commission, Directorate General Joint Research Centre, the Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium. Seeds of non-modified maize and 59122 maize both supplied by Pioneer Hi-Bred International (Johnston, IA, USA) were rinsed with demineralised water, drained and dried at 30 °C in order to minimise dust contamination from other crops. After a two step grinding process, transforming the seeds into a non-modified maize powder and a 59122 GM maize 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. The 59122 mass fractions of ERM®-BF424 were verified with the help of DNA-based detection methods. The CRMs are available in glass bottles containing 1 g of maize powder packed under argon atmosphere.

EUROPEAN COMMISSION
DIRECTORATE-GENERAL
Joint Research Centre

The mission of the Joint Research Centre is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of European Union policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Community. 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.

15 LA-NA-22333-EN-C

ISBN 92-79-02755-7

Publications Office
Publications.eu.int