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CERTIFICATION REPORT

Certification of Reference Materials of Potato Powder with Different Mass Fractions of the Event AM04-1020

Certified Reference Materials ERM®-BF430 (ERM®-BF430a, ERM®-BF430b, ERM®-BF430c, ERM®-BF430d, ERM®-BF430e)

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Certified Reference Materials ERM®-BF430 (ERM®-BF430a, ERM®-BF430b, ERM®-BF430c, ERM®-BF430d, ERM®-BF430e)

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Abstract

This report describes the processing and certification of five potato powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) AM04-1020 potato (ERM-BF430a, b, c, d, e). The materials were prepared and certified in 2010 and 2011 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), according to the principles of ISO Guide 34. The five CRMs have been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

GM tubers of AM04-1020 potato and of a non-GM potato variety were washed and dried. Afterwards they were ground with a cryo-grinding vibrating mill to obtain a GM and non-GM powder base material. Gravimetric mixtures of non-GM and GM potato powder were made by dry-mixing. A first material was made by mixing non-GM and GM potato powder and two additional ones by further dilution of the mixture with non-GM potato powder. The certified values of these CRMs were calculated from the gravimetric preparations, taking into account the respective purity of the base materials and their water mass fractions. Quantification of the mass fraction of AM04-1020 in the powders by quantitative real-time polymerase chain reaction confirmed the dilution process of the gravimetrically prepared ERM-BF430 (measurements within the scope of accreditation to ISO/IEC 17025).

The certified values and uncertainties of the five CRMs are as follows:

CRM	Quantity [1)]	Certified value [g/kg]	Uncertainty [2)] [g/kg]
ERM-BF430a	Mass fraction	0	negligible
ERM-BF430b	Mass fraction	1000	negligible
ERM-BF430c	Mass fraction	10.0	1.2
ERM-BF430d	Mass fraction	40	5
ERM-BF430e	Mass fraction	100	12

[1)] Mass fraction of AM04-1020 potato (unique identifier code BPS-A1Ø2Ø-5) based on the masses of dried genetically modified AM04-1020 potato powder and dried non-genetically modified potato powder, taking into account their respective purity with regard to AM04-1020 and their water content. The certified value is traceable to the International System of Units (SI).

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

The CRMs are intended for the quality control or calibration of methods for the quantification of AM04-1020 potato in food and feed. The CRMs are available in glass bottles containing 1 g of dried potato powder closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.

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GLOSSARY

AM04-1020	GM potato (*Solanum tuberosum)* event AM04-1020
ANOVA	analysis of variance
BPS	BASF Plant Science Company GmbH
CRM	Certified Reference Material
Ct	Cycle threshold
CTAB	cethyl trimethyl ammonium bromide
DNA	deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
ERM®	trademark of European Reference Materials
EURL-GMFF	European Reference Laboratory for Genetically Modified Food and Feed
g	standard gravity
GM	genetically modified
GMO	genetically modified organism
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
KFT	Karl Fischer titration
LOD	limit of detection
m/v	mass per volume
v/v	volume per volume
N	number of samples analysed
n	number of subsamples analysed
n.a.	not applicable
PSA	particle size analysis by laser diffraction
qPCR	quantitative polymerase chain reaction
RT	room temperature
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 real-time PCR
TE	tris hydroxymethyl aminomethane (TRIS) ethylene diamine tetraacetate (EDTA)
U	expanded uncertainty
u^*_{bb}	standard uncertainty related to the between-bottle heterogeneity that can be hidden by the method repeatability
u_{bb}	standard uncertainty related to the between-bottle heterogeneity
u_{char}	standard uncertainty related to the characterisation
ugp	potato-specific UDP-glucose pyrophosphorylase gene
u_{lts}	standard uncertainty related to the long-term stability of the material
$\overline{x}$	calculated mean value

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 methods for GMO quantification, and on the other hand the production of reference materials for the calibration or quality control of these methods.

This certification report describes the development and certification of a set of matrix Certified Reference Materials (CRMs) for the potato event AM04-1020 from non-genetically modified (GM) and GM potato tubers. BASF Plant Science Company GmbH (BPS), Ludwigshafen, DE has developed the GM potato event AM04-1020. In accordance with Commission Regulation (EC) No 65/2004 [2] the potato event AM04-1020 was assigned the unique identifier code BPS-A1Ø2Ø-5.

In 2010, the Institute for Reference Materials and Measurements (IRMM, Geel, BE) was asked by BPS to develop and produce a series of reference materials for the quantification of AM04-1020 potato. The resulting CRM has been named ERM-BF430 and is composed beside a non-GM and a GM pure material of gravimetric mixtures of non-GM and GM potato powder. The first mixture was prepared by dry-mixing non-GM and GM potato powder and two additional mixtures were prepared by further dilution with non-GM potato powder.

2 Participants

- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium (BELAC, 268-TEST)*

* Measurements within the scope of accreditation to ISO/IEC 17025.

3 Base material and processing

3.1 Characterisation of the base materials

For the preparation of the CRMs, BPS supplied conventional potato tubers and AM04-1020 potato tubers to IRMM. After arrival, the potato tubers were stored at (4 ± 3) °C in the dark until use. 60 kg of non-GM potato tubers and 25 kg of AM04-1020 potato tubers were used for the processing of ERM-BF430.

The purity of the delivered batches has been tested by BPS in the frame of quality control measurements. The delivered GM tubers were reported by BPS to contain the AM04-1020 event and to be at least 99.5 % pure (tested at 95 % confidence level). The delivered non-GM tubers were reported by BPS to contain no AM04-1020 event and to be at least 98 % pure (tested at 95 % confidence level).

The purity and genetic composition of these batches were verified at IRMM by analysis of the presence of amylose in the tubers with Lugol's staining solution [3]. Surface chips from conventional potato tubers turn dark blue upon reaction with the iodine solution as they contain about 25 % amylose, while those from the GM tubers, which largely lack amylose, remain orange/brown. Every potato tuber was tested individually for amylose before inclusion in the processing. The results of these assays confirmed that all non-GM tubers contained amylose and therefore lacked the genetic modification. All GM tubers were negative for amylose, indicating the presence of the genetic modification (Table 1).

Table 1: Purity test of the GM and non-GM potato batches used for the production of ERM-BF430, based on a colorimetric detection method for amylose

Batch	Number of tubers used for processing (*N*)	Number of tubers tested (*N*)	Number of amylose positives	Number of amylose negatives
Non-GM	810	810	810	0
GM	241	241	0	241

The genetic identity of the non-GM and GM base material was confirmed at IRMM by quantitative real-time PCR (qPCR) targeting the GM insert junction with the plant genome. Quantitative PCR was performed according to a method delivered under confidentiality agreement between BPS and IRMM. In the future a quantification method for AM04-1020 will be published after validation on the homepage of the European Reference Laboratory for GM food and feed (EURL-GMFF) [4]. Genomic DNA extracted from pure AM04-1020 potato powder was used for calibration. Detection was done on an ABI7900 HT instrument following the TaqMan® Universal PCR Master Mix protocol (Applied Biosystems, Foster City, CA, USA) [5]. PCR analysis on 20 randomly selected non-GM and 20 GM tubers confirmed that no GM contamination was in the non-GM lot, while all GM tubers were confirmed to contain the GM event AM04-1020 (Table 2). The results revealed a complete correspondence between PCR data and results obtained with the amylose test.

Table 2: Purity tests of selected tubers of the GM and non-GM potato batches used for the production of ERM-BF430, based on event-specific qPCR

Batch	Number of tubers used for processing (*N*)	Number of tubers tested (*N*)	Number of transgene positives	Number of transgene negatives
Non-GM	810	20	20	0
GM	241	20	0	20

Additionally the non-GMO and GMO base material has been analysed for the presence of the potato event EH92-527-1. Genetically modified EH92-527-1 potatoes give the same measurement result as AM04-1020 potatoes when tested with Lugol's staining solution. For both, the non-GMO and the GMO base material, the results indicated that no contamination with EH92-527-1 is present. All results with the event-specific qPCR for EH92527-1 were below the LOD of the method of 0.02 g/kg (N = 2, n = 1, analysed in triplicate, data not shown).

3.2 Processing of the base materials

The GM and non-GM base materials were processed separately. Due to the total mass of the non-GMO batch, processing was carried out in two individual batches which were merged later on. Cross-contamination and contamination with foreign DNA were avoided applying systematic cleaning and frequent exchange of the laboratory clothing. All potential contact surfaces were treated with a DNA degrading solution (DNA-Erase™, MP Biomedicals, Irvine, CA, USA) before processing 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 potato tubers received by IRMM were carefully rinsed twice with tap water. Sprouts were removed. The tubers were dried with cotton and starch-free tissues and finally air dried. The tested potatoes were cut with a hand chipping machine CF-5 (Sammic, Azkoitia, ES) into French fries of about 1 cm thickness and then manually cut into cubes of about 1 cm^3. The cubes were then further dried during 86 hours in a freeze drier (Epsilon 2-65D, Osterode, DE). Until further processing the dried potato cubes were packed in portions of 2 kg in plastic bags and stored at -20°C in a container under nitrogen atmosphere.

For milling a cryo-grinding vibrating mill (Palla mill, KHD, Humboldt-Wedag, Köln, DE) was used. Prior to this milling step the potato cubes were frozen for at least 12 hours in metal containers in liquid nitrogen. The mill was also cooled down to process the potato cubes at a temperature below -80 °C. The feeding speed of the mill was set in such a way as to ensure most efficient milling with respect to the particle size obtained. After milling, the powder was kept at room temperature to reach a thermal equilibrium. The GM and non-GM powders were sieved separately using a 500 µm stainless steel mesh on a sieving machine (Russel Finex, London, UK). In case of the non-GM powder a coarse fraction of 25 g did not pass the 500 µm mesh. In case of the GM powder a coarse fraction of 5 g did not pass the 500 µm mesh. Both coarse fractions were removed. The sieved potato powder was mixed using a Turbula mixer (Turbula System Schatz T200B, WAB, Basel, CH) for 60 min to improve equal distribution of the different parts of the potato tissues separated by the milling and sieving process.

For the non-GM and GM powder a residual water mass fraction of (37 ± 6) g/kg and (31 ± 5) g/kg with *U, k* = 2 was measured by volumetric Karl Fischer titration (KFT) (*N* = 1, *n* = 3). The particle volumes were measured based on laser diffraction patterns (PSA, HELOS, Sympatec, Clausthal-Zellerfeld, DE) and compared for both powders (*N* = 1, *n* = 5). They were found to be similar with respect to their particle size distributions (Figure 1). Prior to the dry-mixing the actual water mass fraction was measured. The final water mass fractions can be found in Table 6.

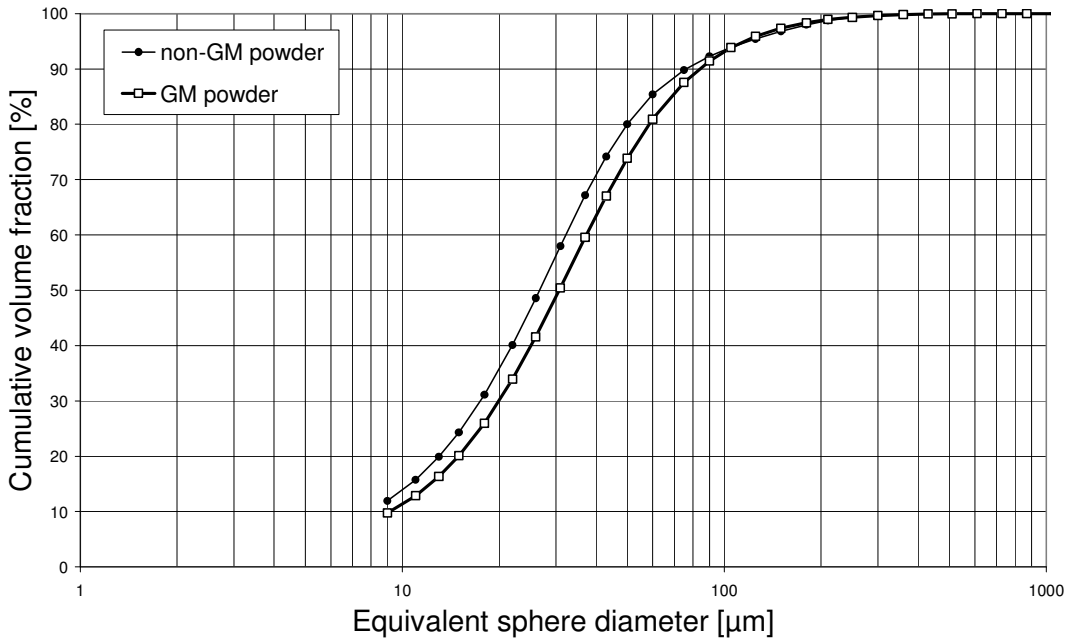

Figure 1: Accumulation of particle volume fractions in the GM powder and non-GM powder analysed by PSA (*N* = 1, *n* = 5). The total volume is set as 100 %.

3.3 Gravimetric preparation of mixtures

The ground base materials were used to produce a blank material for AM04-1020, a pure AM04-1020 material and three mixtures containing different mass fractions of AM04-1020 potato powder in non-GM potato powder at nominal mass fraction levels of 10, 40 and 100 g/kg, respectively. All five materials were treated according to the same procedure. The powder materials were weighed using a calibrated balance with an intermediate precision, expressed as relative standard uncertainty of 0.1 %. Calibration was carried out annually by an external company accredited for ISO/IEC 17025 calibration services; additionally the performance of the balance was verified before use. Weighed portions of the powder materials were homogenised in a propeller mixer for 2 min while being flushed with Argon. The blank material was processed first, followed by the pure GM material. The mixtures were processed after thorough cleaning of the dry-mixer. For the preparation of the mixtures the masses of the non-GM and GM powder were corrected for their respective water mass fractions (Table 6). The material having a nominal mass fraction of 100 g AM04-1020/kg was produced by mixing pure GM with pure non-GM ground base materials. The materials having a nominal mass fraction of 10 and 40 g AM04-1020/kg were produced by further dilution of the 100 g/kg GM powder with pure non-GM powder. The gravimetric preparation formed the basis for the calculation of the mass fraction of the powders (Section 6).

3.4 Filling

The powders were filled in 1 g portions in 10 mL brown glass bottles using an automatic filling device. The first 30 bottles of each batch were discarded as an additional precaution against carry-over contamination. The bottles were automatically closed with rubber lyophilisation inserts. Before final closure of the bottles, the lyophilisation inserts were manually lifted in order to allow air circulation. In a freeze-drier the air was evacuated and replaced by argon. The bottles 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 mass fraction levels of AM04-1020: nominal 0 g/kg = silver (BF430a), nominal 1000 g/kg = black (BF430b), nominal 10 g/kg = red (BF430c), nominal 40 g/kg = pink (ERM-BF430d), nominal 100 g/kg = brown (BF430e), consistent with the cap colours of previous IRMM CRMs for GMOs. Each of the bottles was identified by a numbered label indicating the ERM code (Figure 2). Following the inventorying and the selection of bottles for future analysis according to a random stratified sampling scheme, the bottles were brought to a storage room for long-term storage in the dark at (4 ± 3) °C.

Figure 2: Prototype labels for the ERM-BF430 series. The denotation 'blank' was used for the nominal mass fraction of 0 g/kg AM04-1020 potato powder (BF430a), while BF430b refers to the nominal 1000 g/kg and BF430c, BF430d and BF430e refer to the nominal 10 g/kg, 40 g/kg and 100 g/kg AM04-1020 potato, respectively.

3.5 Water content and particle volume distribution

The residual mass fraction of water in ten randomly selected bottles from each of the powder materials was determined by volumetric KFT. The results are summarised in Table 3 and resemble the final water content in the CRM. It should be noted that the powder materials are hygroscopic.

Table 3: Water mass fraction in candidate CRMs ERM-BF430 determined by volumetric KFT (*N* = 10, *n* = 3)

Candidate CRM	Water mass fraction [g/kg]	
	$\overline{x}$	*U (k = 2)*
ERM-BF430a	15.6	4.0
ERM-BF430b	15.3	3.9
ERM-BF430c	13.6	3.5
ERM-BF430d	18.9	4.8
ERM-BF430e	15.6	4.0

Five randomly selected bottles from each of the powder materials were analysed twice for their particle volume distribution (N = 5, n = 2) based on laser diffraction patterns (PSA, HELOS, Sympatec, Clausthal-Zellerfeld, DE). It is important to understand that the cumulative volume distribution of particles derived from laser light scattering data is based on their equivalent spherical diameter, i.e. the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. Since most particles are presumably not perfectly spherical, the calculated volume of the particles based on their diameter is, therefore, overestimating the average particle size. The mean particle diameters calculated by the PSA software are given in Table 4. The powders had a maximum particle diameter below 430 µm (Figure 3).

Table 4: Mean particle diameter for the candidate CRMs ERM-BF430 determined by PSA (*N* = 5, *n* = 2)

Candidate CRM	Mean particle diameter [µm]	
	$\overline{x}$	*s*
ERM-BF430a	32	2
ERM-BF430b	24	4
ERM-BF430c	29	3
ERM-BF430d	30	2
ERM-BF430e	31	3

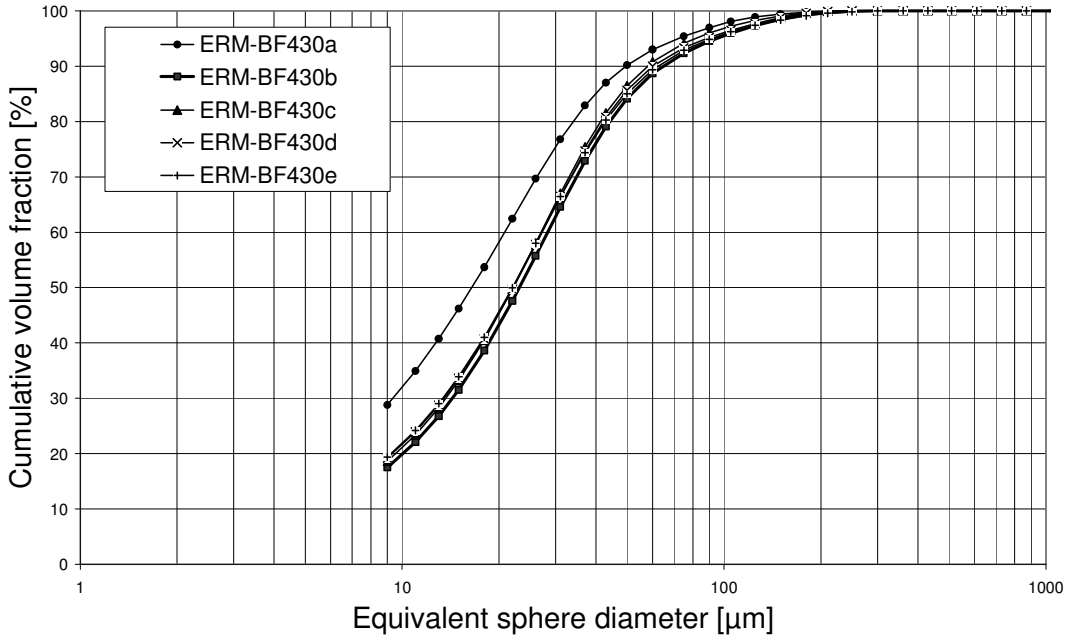

Figure 3: Accumulation of particle volume fractions in ERM-BF430 analysed by PSA (*N* = 5, *n* = 2). The total volume is set as 100 %.

3.6 DNA content of the base materials

Three of the described CRMs are mixtures of GM and non-GM potato powders, produced gravimetrically and intended to be used for calibration or quality control of quantitative measurements of the genomic DNA, following DNA extraction and purification. DNA-based quantification methods are applied by GMO testing laboratories and any DNA mass fraction difference in the non-GM and GM base materials will lead to a shift of the measurement results obtained with e.g. real-time PCR. The mass of DNA in both base materials was estimated by a slight modification of the classical fractionation method developed initially by Ogur & Rosen [6]. A 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 was carried out. The mass of DNA was measured spectrophotometrically after derivatisation with diphenylamine. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases [6, 7]. The extractable DNA mass fraction of the two materials was calculated as:

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

The ratio of the DNA mass extractable from 100 mg of GM and non-GM potato powder was found to be 1.02 ± 0.17 (N = 9). A t-test demonstrated that no significant difference between the DNA mass extracted from the GM and non-GM potato powders by the modified Ogur and Rosen method exists at 95 % confidence level.

3.7 Confirmation measurements

As a control for the gravimetric preparations, the mass fraction of AM04-1020 potato in all five CRMs was verified by the confidential real-time PCR method provided by BPS targeting the insertion region of the transgenic DNA in this potato event and the potato-specific UDP-glucose pyrophosphorylase (*ugp)* gene. Genomic DNA was extracted from 200 mg powder samples using a cethyl trimethyl ammonium bromide (CTAB)/Microspin DNA extraction method (see Annex). The real-time PCR test was calibrated with genomic DNA extracted from the pure AM04-1020 potato powder, and afterwards diluted in a TE buffer solution (pH 8.0, 2 mmol/L TRIS and 0.2 mmol/L EDTA) to produce a calibration curve between undiluted and 200-times diluted for the transgenic gene and target taxon-specific gene. The efficiency of the amplification was determined from the slope of the regression line between the calibrants' mass fractions of AM04-1020 and the obtained Ct-values; for all standard curves, the PCR efficiency was within the control limits set to 72 and 142 %. The limit of detection (LOD) was calculated using the calibration curve approach [8] but estimating *s* on the lowest dilution measured. The results of the quantification of AM04-1020 for the five candidate CRMs are shown in Table 5. Taking the uncertainties into account the qPCR measurements confirm the dilutions applied for the mixtures. However, as no independent calibration was carried out, the data displayed in Table 4 can be used for confirmation of the processing, but do not necessarily resemble the true value. It has to be noted that the calibrant used for the transgenic and the taxon-specific target is genomic DNA extracted from the pure AM04-1020 potato powder, consequently the deviation from the nominal mass fraction values (10, 40 and 100 g/kg) refers to a different extractability pattern or analytical behaviour of the taxon-specific target extracted from the non-GMO material or matrix effects caused by the GMO or the non-GMO material. However, the deviation can also be caused by more than one of these four effects acting simultaneously.

Table 5: Quantification of the AM04-1020 potato mass fraction in the candidate CRMs by event-specific real-time PCR using genomic DNA from pure AM04-1020 powder for calibration

Candidate CRM	AM04-1020 potato mass fraction [g/kg]	U (k = 2) [g/kg]
ERM-BF430a	< 0.6 [1) 2)]	-
ERM-BF430b	842.7 [2)]	77.5
ERM-BF430c	9.7 [3)]	0.9
ERM-BF430d	36.0 [3)]	3.1
ERM-BF430e	95.2 [3)]	8.8

[1)] The obtained value is below the LOD determined during method validation (0.6 g/kg).
[2)] Mean for 3 samples from each of 5 random bottles (N = 5, n = 3), with each sample (n) measured in three replicates.
[3)] Mean for 3 samples from each of 12 random bottles (N = 12, n = 3), with each sample (n) measured in three replicates.

4 Homogeneity

4.1 Homogeneity study

Here we report on the results of a homogeneity study performed on each of the three GM potato mixtures (ERM-BF430c, d, and e). As ERM-BF430a and b are pure materials for which the individual purity has been accessed (Section 3.1), no additional homogeneity study was required.
The degree of homogeneity of the powder in ERM-BF430c, d and e with respect to the mass fraction of AM04-1020 potato was measured by real-time PCR using a random stratified procedure. This homogeneity study was planned together with the measurements to confirm the gravimetric preparations (Section 3.7). The measurement results were obtained under conditions chosen as repeatability as possible, but required several days for DNA extraction and individual calibrations for each PCR plate. Measurements were carried out on bottles randomly taken from the entire batch and analysed in a randomised order (related to their DNA extraction day and the PCR analysis day). Therefore, the results were as well suited to investigate the homogeneity. For ERM-BF430c, d and e data obtained on 3 samples from each of 12 random selected bottles ($N = 12$, $n = 3$) were available. Each sample was measured in three replicates by real-time PCR.

In a first step the data were visually checked using normal probability plots and histograms. No indications were found that the data follow no unimodal distribution. A regression analysis was used to evaluate potential drifts in results related to the analysis sequence based on the order of the DNA extraction, the analysis sequence based on the order of the PCR measurements or to the filling sequence. No significant trends were observed in the results for the filling sequence and the DNA extraction sequence. However, significant trends were observed for the analysis sequence based on the order of the PCR analysis. For ERM-BF430c, d and e the trend was significant at 95 % confidence level and in the case of ERM-BF403d the trend was also significant at 99 % confidence level, pointing at instability of the analytical system. As the analytical sequence and the unit numbers were not correlated, correction for these trends can improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. Therefore, trends in the analytical sequence were corrected as shown below:

$$\text{corrected result} = \text{measured result} - b \cdot i$$

(b = slope of the linear regression; i = position of the result in the analytical sequence)

The trend-corrected and the non-corrected datasets were tested for consistency using Grubbs outlier tests on a confidence level of 95 % on the individual results and the unit means. For both data sets of ERM-BF430e one outlier was detected but was retained as no technical reason to exclude the outlier could be found.

ANOVA statistics were used to calculate the between bottle standard deviation (s_{bb}) and the relative maximum standard uncertainty related to the inhomogeneity that can be hidden by the method repeatability (u^*_{bb}), using the formulas [9]:

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

(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 calculated on the basis of the corrected data sets and converted into relative uncertainties and expressed as percentage (Table 6). The higher values for $s_{bb, rel}$ or $u^*_{bb, rel}$ of each CRM concentration was included in the calculation of the overall uncertainty on the certified values (Section 7.2).

Table 6: Relative standard uncertainties linked to the heterogeneity between bottles of dry-mixed AM04-1020 potato candidate CRMs analysed by real-time PCR

Candidate CRM	$s_{bb, rel}$ [%]	$u^*_{bb, rel}$ [%]
ERM-BF430c	6.3	5.0
ERM-BF430d	4.6	5.3
ERM-BF430e	n.a. [1)]	5.2

[1)] As MS_{bb} was smaller than MS_{wb}, s_{bb} could not be calculated.

4.2 Minimum sample intake for analysis

The potato DNA extraction method employed here uses 200 mg sample intake. Within the frame of the gravimetric confirmation and homogeneity measurements (Section 3.7 and 4.1), it was shown that reliable PCR results can be obtained if 200 mg sample intake is used for DNA extraction. The minimum amount of sample to be used is 200 mg.

Because of the hygroscopic nature of the powders, it is recommended to close the bottles immediately after taking a sample. It should however be noted that possible water uptake does not influence the certified value but might favour the growth of microorganisms in the powder materials.

5 Stability

5.1 Short-term stability

The short-term stability of ERM-BF430 was investigated following isochronous incubation of bottles at 4, 18 and 60 °C for 1, 2 and 4 weeks. ERM-BF430e was chosen for this study (nominal 100 g/kg) as it contains the highest AM04-1020 content of the mixed materials and therefore was found best suitable to detect possible instabilities. From each of the 5 bottles per condition (N = 5), two samples (n = 2), were analysed for stability of the DNA in the matrix. A similar number of reference samples were likewise analysed, which were kept at the reference temperature of -70 °C during the 4 weeks. The same DNA extraction and real-time PCR method was used as for the verification of the mass fraction (Section 3.5) and the homogeneity (Section 4.1).

No outliers were detected in the whole data sets using the single and double Grubbs tests at a 95 % level of confidence. Regression analysis revealed no trend over the time period of 4 weeks for the samples incubated at 4, 18 and 60 °C (t-test, 95 % level of confidence). Based on these results and the existing experience about the stability of potato powders, it was concluded that the uncertainty due to degradation during dispatch is negligible for all five candidate CRMs. ERM-BF430 can be shipped under ambient conditions.

5.2 Long-term stability

The long-term stability of a similarly produced potato powder CRM (ERM-BF421b) has been investigated earlier. ERM-BF421b and ERM-BF430 have both been produced from GM potato tubers which largely lack amylose, whereas the non-GM tubers contained amylose. It therefore appears applicable to use long-term stability data generated on ERM-BF421b for the estimation of the standard uncertainty contribution of the long-term stability for ERM-BF430.

An isochronous incubation of bottles at 4 and 18 °C for 3 and 6 months was carried out for ERM-BF421b (N = 10) [10]. Additionally stability monitoring data for the time points 12, 20 and 40 months were available and used to estimate the possible uncertainty contribution of the long-term stability for ERM-BF430. Accepting a shelf life of 24 months, a relative standard uncertainty contribution ($u_{lts,\,rel}$) of 2.3 % for the certified GM content was obtained, which was used as an estimation of the standard uncertainty contribution for the stability of the CRM ERM-BF430 (Section 7.2).

An intensive post certification monitoring is carried out to check the stability of ERM-BF430 at regular intervals of 1 year.

It is generally recommended to store bottles of ERM-BF430 in the dark and within the temperature interval of (4 ± 3) °C.

6 Characterisation

The five candidate CRMs under the label ERM-BF430 are potato powder materials processed from non-GM and GM tubers. While ERM-BF430a is prepared from the pure blank material and ERM-BF430b from pure AM04-1020 material, the other CRMs of the ERM-BF430 series are gravimetrically produced mixtures of the pure non-GM and GM powders. ERM-BF430 is being certified for its AM04-1020 mass fraction.

The mass values are based on the mass fractions of dry-mixed GM and non-GM powders, corrected for their water mass fractions, and taking into account the powder's purity with regard to the AM04-1020 event. The values were calculated according to the following formulas:

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

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

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

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

In Table 7, the data supporting the calculation of the mass fractions of AM04-1020 are summarised.

Table 7: Subsequent mixing of GM AM04-1020 potato powder with non-GM powder to prepare ERM-BF430 materials

ERM code [1]	GM powder			Non-GM powder [2]	Mixtures
	Mass fraction of GM powder [g/kg]	Water mass fraction ± *U* (*k* = 2) [g/kg]	Mass [g]	Mass [g]	Resulting mass fraction of GM powder [g/kg]
BF430a	n.a.	n.a.	0	4000.0	0.0
BF430b	1000.0	30.5 ± 4.3	4000.0	0	1000.0
BF430e	1000.0	30.5 ± 4.3	395.4	3602.4	100.0
BF430d	100.0 [3]	37.4 ± 5.3	1599.0	2398.2	40.0
BF430c	100.0 [3]	37.4 ± 5.3	398.6	3599.0	10.0

[1] Rows in the order of the processing.
[2] The non-GM powder used for the gravimetric preparations had a water mass fraction of 40.3 ± 10.3 g/kg (*U, k* = 2) and was considered to be free of AM04-1020 potato.
[3] For the preparation of BF430c and d, the 100 g/kg GM powder was used.

7 Certified values and uncertainty budgets

7.1 Certified value

The ERM-BF430 series is composed of five CRMs certified for the mass fraction of AM04-1020 potato powder. The certified values are based on the masses of dried powder of GM tubers and non-genetically modified tubers used in the gravimetrical preparation. The masses of the powders were corrected for their respective water mass fractions during the preparation of the materials (Section 6).

The purities of the GM and non-GM batches used for the processing of these powders were investigated in order to be able to calculate the certified value. No indication was found that the GM AM04-1020 potato base material contained tubers being negative for the event AM04-1020 (Section 3.1). No indication for the presence of AM04-1020 was found in the non-GM powder by real-time PCR (Table 5). As no evidence for a contamination was found in either of the two base materials and as every potato entering the processing has been checked beforehand, 100 % purity was used for the calculation of the certified mass fraction of AM04-1020 in the powder mixtures.

Real-time PCR measurements demonstrated furthermore that no mixing errors were made (Table 5).

As no proof could be delivered that the certified GM powder mass fractions are equal to the corresponding transgenic and target taxon-specific DNA copy number ratio, the user is reminded that IRMM only certifies these materials for their mass fraction of AM04-1020. The DNA content of the two base materials used for producing ERM-BF430 influences the DNA ratio measured by PCR, as well as differences in DNA extraction and/or PCR amplification behaviour for the non GM and GM material. Consequently one has to be careful to draw quantitative conclusions (in gene copy numbers, for instance) from measurements on unknown samples as DNA- and/or protein-based quantification of GMOs may vary with the particular matrix and the potato variety tested [11].

7.2 Uncertainty budget

The expanded uncertainty of the certified value (U_{CRM}) comprises standard uncertainty contributions from the characterisation, the inhomogeneity, and the stability:

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2}$$

The individual uncertainty contributions are summarised in Table 7.

The u_{char} on the certified mass fraction of AM04-1020 was composed of several contributions, i.e. the uncertainty on the mass determination ($u_{char,1}$) and the uncertainty on the water mass fraction analysis ($u_{char,2}$). All potato tubers were checked individually before entering the processing, therefore the uncertainties related to the purity of the non-GM and GM base powders was considered negligible (Section 3.1). A coverage factor of 2 ($k = 2$) was used to calculate the expanded uncertainty corresponding to a level of confidence of about 95 % [12]. The individual standard uncertainty and expanded uncertainty estimations can be found in Table 8.

The uncertainty introduced by the inhomogeneity has been estimated as the relative maximum heterogeneity potentially hidden by the method repeatability ($u^*_{bb,\ rel}$) or the relative between bottle heterogeneity ($s_{bb,\ rel}$) as defined in Section 4.1.

The uncertainty contribution from the long-term stability (u_{lts}) has been estimated on the basis of stability tests on another potato CRM having similar physical properties as ERM-BF430 and was calculated for 24 months (Section 5.2).

Table 8: Uncertainty budgets for the mass fractions of AM04-1020 potato in ERM-BF430

ERM	Certified value [g/kg]	Standard uncertainty contribution [g/kg]				Expanded uncertainty [5] U (k = 2) [g/kg]
		$u_{char,1}$ [1]	$u_{char,2}$ [2]	u_{bb} [3]	u_{lts} [4]	
BF430a	**0**	n.a.	n.a.	n.a.	n.a.	-
BF430b	**1000**	n.a.	n.a.	n.a.	n.a.	-
BF430c	**10**	0.0135	0.0906	0.6287	0.2295	1.4
BF430d	**40**	0.0468	0.3630	2.1200	0.9200	5
BF430e	**100**	0.0959	0.7395	5,1903	2.2957	12

[1] Standard uncertainty of the mass determination mainly based on the uncertainty of the balance and the number of weighing steps required.
[2] Standard uncertainty of the water mass fraction determination by volumetric KFT.
[3] Standard uncertainty contribution resulting from the homogeneity assessment.
[4] Standard uncertainty resulting from the stability study of dried potato powders during storage at 4 °C, estimated for 24 months.
[5] Rounded expanded uncertainties are given.

8 Metrological traceability and commutability

8.1 Metrological traceability

The ERM-BF430 series is composed of five reference materials certified for the mass fraction of event AM04-1020 potato powder. The certified values are based on gravimetric dry-mixing of non-modified potato powder with event AM04-1020 potato powder. The respective certified value is traceable to the International System of Units (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 tubers and the water content of the powder at the time of the gravimetric mixing has been taken into account when calculating the certified value.

8.2 Commutability

ERM-BF430 is prepared from non-GM and GM potato powder. DNA extracted from ERM-BF430 and targeted by the event-specific PCR method is genomic DNA. The type of DNA is therefore identical to the DNA extracted from food and feed samples and targeted by PCR. Provided a suitable DNA extraction and purification method is chosen, which is leading to a genomic DNA quality and purity suitable for PCR amplification, commutability [13] problems can be excluded.

9 Intended use and instructions for use

The ERM-BF430 series of CRMs is intended for use as quality control material or calibrant in DNA-based methods for the detection of genetically modified material in food and feed.

The minimum amount of sample to be used is 200 mg.

Bottles should be stored in a dry and dark place at (4 ± 3) °C. The materials are hygroscopic. The user is advised to close bottles immediately after taking a sample for analysis.

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Annex (DNA extraction method)

Principle

The CTAB/MicroSpin DNA extraction method employed releases the DNA from the potato matrix into an aqueous solution by thermal lysis in the presence of CTAB, EDTA (Ethylene diamine tetraacetic acid) sodium salt and proteinase K. This is followed by removal of RNA by digestion with RNase A and removal of proteins and lipids by extraction with chloroform. The crude DNA extract is subsequently precipitated with isopropanol. Remaining inhibitors are removed from the suspended DNA extract by filtration through a S-300 HR MicroSpin column (Amersham Pharmacia Biotech, Roosendaal, NL).

Reagents

CTAB buffer:
2 % (m/v) CTAB
1.4 M NaCl
0.1 M Tris base
15 mM Na_2EDTA
adjusted to pH 8.0 with HCl

TE buffer:
2 mM Tris (pH not rather 8.0?)
0.2 mM EDTA (pH 8.0)

Glycogen 20 mg/mL

75 % (v/v) Ethanol

Extraction procedure

Cell lysis and DNA precipitation
(1) Weigh 200 mg of freeze-dried plant potato powder into a 2 mL microcentrifuge tube
(2) Add 1 mL CTAB buffer and 4 µL proteinase K to the tube and mix thoroughly
(3) Incubate for 1 h at 50 °C with agitation
(4) Spin down at room temperature (RT) for 10 min at 13000 *g*
(5) Transfer the supernatant to a microcentrifuge tube containing 5 µL RNase A
(6) Incubate 15 min at 60 °C with agitation
(7) Centrifuge at RT for 1 min at 13000 *g*
(8) Transfer the supernatant to a microcentrifuge tube containing 500 µL chloroform
(9) Vortex and centrifuge at RT for 10 min at 13000 *g* to separate the phases
(10) Transfer the upper phase to a microcentrifuge tube containing 800 µL isopropanol and 2 µL of glycogen into the lid of the tube to enhance the precipitation of the DNA (do not mix the glycogen with the isopropanol on beforehand)
(11) Mix by inverting the tube several times and allow precipitation at RT for 30 min
(12) Centrifuge at RT for 10 min at 13000 *g*
(13) Discard supernatant
(14) Add 500 µL 75 % (v/v) ethanol and pipette carefully up and down until the pellet is detached from the wall of the tube
(15) Centrifuge at RT for 5 min at 13000 *g*
(16) Remove and discard the supernatant
(17) Spin shortly and remove all remaining ethanol. If any fluid remains, allow the pellet to dry at RT
(18) Suspend the pellet in 100 µL TE buffer

(19) Leave for 15 min at RT with occasional agitation
(20) Spin down for 2 min at 13000 *g*
(21) Transfer the supernatant into a new tube
(22) Prepare MicroSpin column according to the manufactur's description and centrifuge at RT for 2 min at 735 *g* to obtain the purified DNA

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Abstract

This report describes the processing and certification of five potato powder Certified Reference Materials (CRMs) containing different mass fractions of genetically modified (GM) AM04-1020 potato (ERM-BF430a, b, c, d, e). The materials were prepared and certified in 2010 and 2011 by the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM, Geel, Belgium), according to the principles of ISO Guide 34. The five CRMs have been accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.
GM tubers of AM04-1020 potato and of a non-GM potato variety were washed and dried. Afterwards they were ground with a cryo-grinding vibrating mill to obtain a GM and non-GM powder base material. Gravimetric mixtures of non-GM and GM potato powder were made by dry-mixing. A first material was made by mixing non-GM and GM potato powder and two additional ones by further dilution of the mixture with non-GM potato powder. The certified values of these CRMs were calculated from the gravimetric preparations, taking into account the respective purity of the base materials and their water mass fractions. Quantification of the mass fraction of AM04-1020 in the powders by quantitative real-time polymerase chain reaction confirmed the dilution process of the gravimetrically prepared ERM-BF430 (measurements within the scope of accreditation to ISO/IEC 17025).
The certified values and uncertainties of the five CRMs are as follows:

CRM	Quantity [1)]	Certified value [g/kg]	Uncertainty [2)] [g/kg]
ERM-BF430a	Mass fraction	0	negligible
ERM-BF430b	Mass fraction	1000	negligible
ERM-BF430c	Mass fraction	10.0	1.2
ERM-BF430d	Mass fraction	40	5
ERM-BF430e	Mass fraction	100	12

1) Mass fraction of AM04-1020 potato (unique identifier code BPS-A1Ø2Ø-5) based on the masses of dried genetically modified AM04-1020 potato powder and dried non-genetically modified potato powder, taking into account their respective purity with regard to AM04-1020 and their water content. The certified value is traceable to the International System of Units (SI).
2) The certified uncertainty is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

The CRMs are intended for the quality control or calibration of methods for the quantification of AM04-1020 potato in food and feed. The CRMs are available in glass bottles containing 1 g of dried potato powder closed under argon atmosphere. The minimum amount of sample to be used is 200 mg.

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