Institute for Reference Materials and Measurements

ERM European Reference Materials

CERTIFICATION REPORT

Certification of EH92-527-1 Fraction and Identity of Non-Modified and Genetically Modified Potato Powder

Certified Reference Materials ERM®-BF421a and ERM®-BF421b

Report EUR 22291 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.

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Certified Reference Materials ERM®-BF421a and ERM®-BF421b

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Summary

This report describes the preparation and certification of the potato powder Certified Reference Materials (CRMs) ERM-BF421a and ERM-BF421b, consisting of conventional and genetically modified EH92-527-1 potato powder, respectively.

The CRMs were processed in 2005/2006 and were certified in 2006 by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium.

The EH92-527-1 genetic modification results in tubers containing nearly exclusively amylopectin (> 98 %) as the starch component, whereas conventional potatoes have starch composed of both amylose and amylopectin in a ratio of 1:3.

For the processing of the CRMs, tubers of non-modified potato and EH92-527-1 potato, supplied by BASF Plant Science GmbH (Ludwigshafen, DE), were individually tested for the presence of the genetic modification, then brushed under tap water, cut into cubes and freeze-dried. After grinding, a pure non-modified potato powder and a pure EH92-527-1 GM potato powder were obtained. The certification of the CRMs was based on the individual testing of the potato tubers used for the production of the CRMs, using an amylose test as a measure for the presence of the genetic modification, and was furthermore confirmed by real-time PCR analysis of the CRMs. The identity of the EH92-527-1 specific modification was also confirmed by nucleotide sequence analysis of the junction region between the plant DNA and the genetic insertion.

The CRMs are intended to be used as positive and negative controls for the detection of genetically modified EH92-527-1 potato in food and feed. The CRMs are available in glass bottles containing 1 g (ERM-BF421a) or 0.5 g (ERM-BF421b) of potato powder, closed under argon atmosphere.

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Glossary

$\bar{x}$	average
Amylopectin	branched (α-1,4 and α-1,6 linkages) glucose polymer in starch
Amylose	linear α-1,4 glucose polymer in starch
AOTF-NIR	acousto optical tunable filter near infrared spectrometry
Bp	basepairs (nucleotide pairs)
Copy number ratio	ratio between the GM DNA copy number and the copy number of the endogenous potato reference gene DNA (UGP)
CRM	certified reference material
CTAB	cetyltrimethylammonium bromide
Ct-value	number of PCR cycles to pass a set fluorescence threshold
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EH92-527-1	GM potato line EH92-527-1; cultivar trade name “AMFLORA”
ERM®	European Reference Materials trademark
GM	genetically modified
GMO	genetically modified organism
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
Kb	kilobasepairs (1000 nucleotide basepairs)
KFT	Karl Fischer titration
LOD	limit of detection
M	molecular DNA marker containing DNA fragments of defined length
M/v	mass per volume
N	number of samples analysed
n	number of subsamples analysed
NIR	near infrared
PCR	polymerase chain reaction
PLS1	partial least squares 1 algorithm (multivariate data analysis)
PSA	particle size analysis by laser diffraction
Rpm	rotations per minute
rt-PCR	real-time PCR
s	standard deviation
T-DNA	transfer DNA, i.e. the DNA region of the Ti plasmid of *Agrobacterium tumefaciens* that is transferred to the plant genome during genetic transformation
U	expanded uncertainty
UGP	uridine diphosphoglucose pyrophosphorylase gene from *Solanum tuberosum*

1 Introduction

Legislation in the European Union demands the labelling of food and feed products 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 development and validation of reliable detection methods, and on the other hand the development and production of reference materials to control the correct application of the detection methods. Therefore, powders have been prepared from genetically modified (GM) potatoes and from non-GM potatoes and these were made available as Certified Reference Materials (CRMs).

The two CRMs (ERM-BF421a and ERM-BF421b) are available from IRMM and its authorised distributors [2]. ERM-BF421 has been accepted as European Reference Material® (ERM®) after successful peer evaluation by the partners of the European Reference Materials concept [3].

According to Commission Regulation (EC) No 65/2004 [4] the event EH92-527-1 potato corresponds to the unique identifier BPS-25271-9.

2 CRM preparation

2.1 Characterisation of the base materials

For the preparation of the CRMs, BASF Plant Science GmbH (Ludwigshafen, DE) supplied tubers of conventional starch potato cultivar KURAS and GM EH92-527-1 starch potato cultivar AMFLORA to IRMM. Approximately 75 kg of non-modified potato and 35 kg of EH92-527-1 potato were used for the processing of ERM-BF421a and ERM-BF421b respectively. The GM potatoes are derived from the tetraploid starch potato cultivar PREVALENT through *Agrobacterium tumefaciens* mediated transformation; they contain a single copy of the T-DNA and have consistently been propagated vegetatively (no sexual hybridisation). The non-modified comparator variety (KURAS) is a different tetraploid starch potato variety. The genetic modification in EH92-527-1 results in the overall biosynthesis of amylopectin as the starch component, whereas conventional potatoes contain starch composed of both amylopectin and amylose in a mass ratio of roughly 3:1.

Quality controls carried out by BASF Plant Science Sweden AB (Svalöv, SE) using event-specific real-time PCR [5] revealed that the genetic modification EH92-527-1 was absent in all 50 randomly selected potatoes from the non-GM batch, and that it was present in all 50 randomly selected tubers from the GM batch. Using statistical calculations, it was concluded that both batches were at least 94 % genetically pure (95 % confidence interval).

The purity and genetic composition of these batches were further verified at IRMM by analysis of the presence of amylose in the tubers with Lugol's staining solution [6]. Surface chips from conventional potato tubers turned dark blue upon reaction with the iodine solution, while those from the GM tubers, which largely lack amylose, remained yellow/orange (Figure 1). 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, and all GM tubers had undetectable amylose, indicative for the presence of the GM event (Table 1). Remark further that the GM potatoes had a more yellow-coloured interior than the non-GM potatoes (see Figure 1), adding an easy visual discrimination aspect during processing.

Figure 1: Amylose staining of potato tubers. Tuber chips (from the surface of the tuber and from the starchy tissue below) were cut from non-GM (left) and GM potatoes (right), then stained with Lugol's solution and photographed after 1 minute. Blue colour indicates presence of amylose.

Table 1: Purity test and genetic composition of the GM and non-GM potato tuber batches used for the production of ERM-BF421, based on the presence of amylose

Batch	Number of tubers used for processing (N)	Number of tubers tested (N)	Number of amylose positives[1]	Number of amylose negatives[1]
Non-GM	549	549	549	0
GM	493	493	0	493

[1] Conventional potato starch contains about 25 % amylose and scores positive in the amylose staining test, while EH92-527-1 potato starch has < 2 % amylose [5] and stains negative in the same test

Additionally, the genetic identity of the non-GM and GM base material was tested at IRMM by qualitative PCR targeting the GM insert junction with the plant genome and the endogenous UGP gene as a positive control. PCR analysis on 10 randomly selected non-GM and 10 GM tubers indicated that no GM contamination was detected in the non-GM lot, while all GM tubers were confirmed to contain the GM event EH92-527-1 (Figure 2). The mass of genomic DNA used in the PCR test was 12 ng per reaction, and the sizes of the amplification products corresponded to the expected sizes of the sequences spanned by both primer pairs. The same tubers analysed by PCR were also tested for the presence of amylose, and the results revealed a complete correspondence between PCR data and results obtained with the amylose test (not shown).

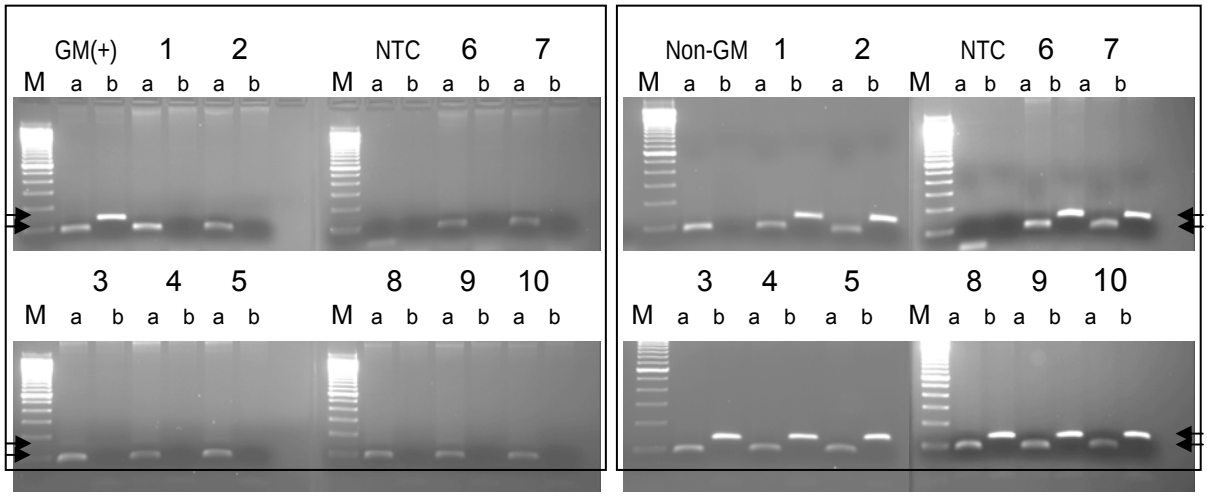


Figure 2: Qualitative PCR analysis for GM event EH92-527-1 and the UGP reference gene on genomic DNA extracted from non-GM (left) and GM potato tubers (right).

Genomic DNA purified from 10 non-GM and from 10 GM potatoes (numbered 1-10 each) was subjected to PCR. Lane a, UGP reference gene (88 basepairs, lower arrow); lane b, GM event EH92-527-1 (134 basepairs, upper arrow). The positive control consisted of DNA isolated from a GM potato (for analysis of non-GM potatoes, left image) or DNA from a non-GM potato (for analysis of GM potatoes, right image). Negative controls were “no template controls” (water). M, 100 bp DNA ladder.

2.2 Processing of the base materials

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

Whole tubers were used for the processing, as 1) preliminary studies showed that the peel contained much more DNA than the potato flesh (per dry mass), and 2) the starch processing industry also uses unpeeled potatoes for the extraction of starch. The tubers processed were individually tested for amylose by cutting a ~1 cm^2 chip from the tuber surface and applying a droplet of Lugol's solution [6] onto the cut surface. Colour development was scored as positive or negative after 1 minute. The tubers were brushed and rinsed under tap water to remove dust and remaining soil and drained. After cutting away any deteriorated parts, the tubers were cut into cubes of about 0.8 cm^3 and freeze-dried for 133 h primary drying and 2-10 h secondary drying, resulting in a 2/3 mass fraction loss. The dried tuber cubes were then cooled in liquid nitrogen and ground with a vibrating cryogenic mill to obtain the ground base material. The mill was flushed with liquid nitrogen until a temperature of -196 °C was reached, after which the liquid nitrogen was shut off during milling. As the powders were rather hygroscopic, they were kept in plastic bags closed under argon atmosphere and stored in containers at -30 °C until filling into 10-mL vials.

Because the GM base material contained some larger particles (derived from the peel), the powder was sieved through a stainless steel mesh of 0.5 mm before filling. The mass of the sieved-out particle fraction was negligible compared to the total mass of the powder (0.2 % mass fraction), and it was, therefore, decided to discard it.

2.3 Bottling

The ground powders were filled in cleaned 10-mL amber glass vials under room temperature conditions using an automatic filling device. The first 30 bottles of each batch were discarded as an additional precaution against carry-over contamination. Colour-coded caps were used for easy identification: silver for GM, black for non-GM materials. Because special precautions had to be taken due to the hygroscopic nature of the material, filling took place in an automatic filling machine contained in a glove-box filled with argon. For the non-GM potatoes, which were bottled first, a minimum of 1 g of material was filled per vial. For the GM potatoes, bottled several weeks later, a minimum 0.5 g of material was filled. The filled amount was checked at regular intervals on an external balance. Moreover, a nozzle flushed the vials just prior to filling and lyophilisation inserts were pressed into the neck of the vial after filling to maintain the inert atmosphere. Next, the vials were placed on an automatic capping and labelling assembly respecting the fill-order.

2.4 Processing control

2.4.1. Water content

Prior to capping, an acousto optical tunable filter near infrared spectrometer (AOTF-NIR) recorded a NIR spectrum from every vial. The spectra were evaluated using a PLS1 regression model based on calibrants containing mass fractions of 5 to 85 g/kg H_2O in a meat powder, which allowed the estimation of the water content in each vial. The meat calibrants were previously shown to generate water mass fraction data for various matrices that corresponded very well with similar data determined by Karl Fischer titration (KFT). For potato, the trueness of the NIR measurements in comparison with KFT data was also very good (Table 2), and the results showed that both CRMs had a low water mass fraction

(< 17.4 g/kg). Thanks to the NIR measurements it could also be seen that the water mass fraction remained constant over the fill series for both potato materials (data not shown).

Table 2: Water mass fraction in non-GM and GM potato powders determined by AOTF-NIR and volumetric KFT

Material	Method	Water mass fraction [g/kg]	
		$\bar{X}$	*s*
Non-GM potato	AOTF-NIR (*N* = 7021)	17.4	1.5
	KFT[1] (*N* = 2, *n* = 4)	16.4	0.8
GM potato	AOTF-NIR (*N* = 12777)	13.5	3.5
	KFT[1] (*N* = 2, *n* = 2)	11.5	1.8

[1] Measured on the ground base powder before filling

2.4.2 Particle size analysis

The resulting powders were checked for particle size distribution by sieve analysis following ISO 3310-1 using sieves with meshes of 32, 45, 63, 90, 125, 180, 250, 355, 500 and 710 µm. The contents of a number of randomly selected bottles were merged to reach the required sample intake of 10 g for sieving analysis (10 bottles for ERM-BF421a and 20 bottles for ERM-BF421b). The results (Table 3) showed that > 50 % of the mass fraction of particles in both powders had a particle size below 45 µm and > 95 % of the mass fraction consisted of particles < 180 µm. A few large particles (> 710 µm) were observed in both CRMs, but the mass fraction contribution of these was negligible and therefore not reflected in Table 3.

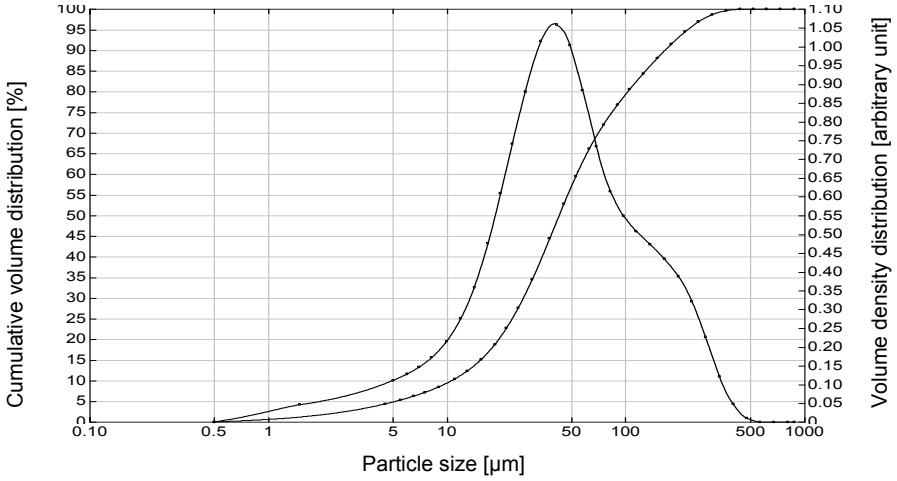
Table 3: Particle size distribution in ERM-BF421 determined by sieving test using a sample intake of 10 g (*N* = 1)

Particle size fraction	Mass fraction of particles according to their size [%]	
	ERM-BF421a	**ERM-BF421b**
< 32 µm	24.44	13.12
< 45 µm	59.25	50.83
< 63 µm	76.59	82.24
< 90 µm	84.33	89.98
< 125 µm	91.37	94.56
< 180 µm	95.83	97.39
< 250 µm	98.31	99.17
< 355 µm	99.70	99.25
< 500 µm	99.93	100.00
< 710 µm	100.00 [1]	100.00 [1]

[1] In both CRMs, some large particles were observed which were > 710 µm in size (more in ERM-BF421a than in ERM-BF421b), but their total mass was below the LOD.

Particle size distribution was also analysed using low angle laser light diffraction. Five randomly selected bottles from each of the powders were used for particle size measurements (N = 5) with a particle size analyser (PSA, Sympatec, Clausthal-Zellerfeld, DE). From each bottle, 3 subsamples were taken and analysed (n = 3). The resulting average particle size distribution curves showed a median particle size of 43 µm for both CRMs (Figure 3) and a maximum particle size below 515 µm. Remark that one of the 15 subsamples from both CRMs showed the presence of coarse particles exceeding the measuring range (0.5-875 µm), which corresponds to the observed large particles mentioned above; as mentioned before, their mass contribution was, however, negligible (the data from these subsamples were omitted from the figures below).

(a)



(b)

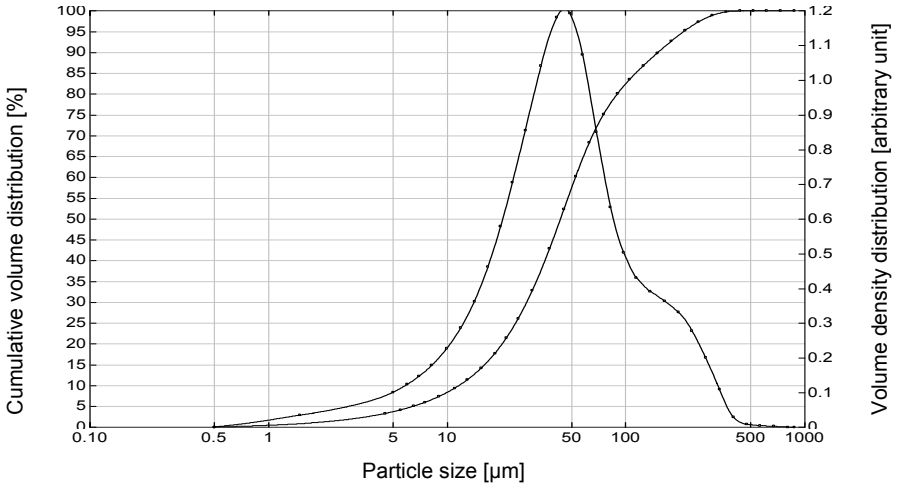


Figure 3: Particle size distribution in ERM-BF421a (a) and ERM-BF421b (b). Average of the measurements performed (N = 5, n = 3).
The cumulative volume distribution of particles derived from laser light scattering data (left Y axis) is based on the maximum diameter of the particles derived from the volume occupied upon rotation of the particles. These data were converted into a volume density distribution curve (right Y axis) showing the distribution of particles in a particular size class, expressed in an arbitrary unit.

2.4.3 DNA mass fraction in both CRMs

To verify the DNA mass fraction in both powders, a slight modification of the classical fractionation method developed initially by Ogur & Rosen was employed [7]. 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 [7, 8]. The ratio between the extractable DNA mass fractions of the two materials was calculated as:

$$\frac{\text{Extractable mass of DNA in 1 g GM potato powder}}{\text{Extractable mass of DNA in 1 g non - GM potato powder}}$$

The results (Table 4) suggested that the DNA mass fraction in the GM potato powder slightly exceeded that in the non-GM potato powder, but a *t*-test showed that the difference was not significant (95 % confidence interval).

Table 4: Ratio of ethanol-precipitating DNA mass fraction in GM and non-GM potato powder

DNA extraction method	***N***	**Mass fraction ratio** $\overline{X} \pm U$ **(*k* = 2)**
Modified Ogur & Rosen [7]	9	1.16 ± 0.28

The DNA mass fraction in both CRMs was also assessed using a CTAB extraction method [10], which is one of several widely used methods for extracting genomic DNA from plants and plant-derived products. Despite reports in the literature on specific DNA extraction methods for potato, we obtained the best results for this CRM using an in-house modified CTAB procedure (Table 5). It should be emphasized that the ERM-BF421 powders, being derived from starch potatoes, obviously have a high starch content; when aqueous suspensions of such powders are heated above 55 °C, the starch swells, resulting in a viscous or gelatinous solution. Furthermore, the modified starch composition in ERM-BF421b, which is nearly exclusively composed of the branched glucose polymer amylopectine, had an increased swelling ability compared to the non-modified potato powder in ERM-BF421a. The presence of the modified starch in the GM powder affected its DNA extractability. This can be seen in Table 6, revealing that twice as much DNA was extracted from the non-GM powder compared to the GM powder. The addition of a starch-degrading enzyme during lysis resulted in a strongly increased DNA recovery for both powders, but a difference in DNA yield remained.

Because of the observed differences in the mass fraction of extractable genomic DNA between ERM-BF421a and ERM-BF421b using the CTAB method, the customer is strongly advised not to prepare gravimetric mixtures of these CRMs and to use the purified DNA from such mixtures for calibration purposes. Depending on the method employed, it should indeed not be assumed that gravimetric mixtures between the CRMs would correspond to equivalent DNA fractions expressed as relative haploid genome copy numbers (see also Section 7 below).

Table 5: DNA extraction protocol for potato ERM-BF421

Step	Details
Weighing	200 mg (ERM-BF421a) or 150 mg (ERM-BF421b) powder
Lysis	Add 1 mL CTAB extraction buffer[1] at 65 °C, 10 µL RNase A (100 mg/mL) and 20 µL Proteinase K (20 mg/mL), mix by pipetting up and down and shaking[2]
	Incubate 30 min at 65 °C with continuous agitation and regular inversion of the tubes, ensuring adequate mixing of the suspension
	Spin down cell debris by centrifugation (10 min, 13000 rpm) and decant supernatant into a new tube
Chloroform extraction (2X)	Add an equal volume of chloroform, mix, centrifuge to separate phases, transfer upper phase to a new tube; repeat once
DNA precipitation	Add 2.2 volumes CTAB precipitation buffer[3], incubate 1 h at room temperature, spin down DNA pellet (10 min, 13000 rpm) and discard supernatant
Resuspension + chloroform extraction	Resuspend DNA pellet in 400 µL 1.2 mol/L NaCl, extract once with equal volume of chloroform, centrifuge to separate phases, transfer upper phase to new tube
Ethanol precipitation	Add 2 volumes cold (-20 °C) ethanol, mix well, and incubate at -20 °C if no precipitate seen (at least 10 min); pellet DNA by centrifugation, wash with cold 70 % (v/v) ethanol and air-dry the pellet
Resuspension	Resuspend the DNA in nuclease-free water, preferably by overnight incubation at 4 °C, then agitate 30 min at 37 °C before use[4]

[1] CTAB extraction buffer: 1.4 % (m/v) CTAB, 1 mol/L NaCl, 0.1 mol/L Tris-HCl, 0.15 mol/L Na_2EDTA, pH 8.0
[2] Although not necessary, the addition of 1 µL of a heat-stable α-amylase (e.g. Sigma # A3403, 786 units/mg) reduces starch swelling and results in an increased DNA recovery (see Table 6)
[3] CTAB precipitation buffer: 0.5 % (m/v) CTAB, 0.04 mol/L NaCl, 0.05 mol/L Tris-HCl pH 8.0
[4] Further purification of the DNA extract, e.g. on a silica or ion-exchange column, may be necessary for certain applications

Table 6: Mass fraction of extractable genomic DNA in ERM-BF421a and ERM-BF421b using the modified CTAB extraction procedure

CRM	DNA extraction method	Sample intake [mg]	No. of samples (N)	DNA mass fraction $\bar{X} \pm s$ [µg/g CRM powder][3]
ERM-BF421a	CTAB	200	68	20.3 ± 3.8
	CTAB + α-amylase[1]	200	15	31.2 ± 2.6
ERM-BF421b	CTAB[2]	150	12	9.5 ± 2.6
	CTAB + α-amylase[1]	150	30	21.6 ± 5.0

[1] One µL of α-amylase (Sigma # A3403) was included during the lysis step
[2] Deviating from the standard protocol, 1.2 mL CTAB extraction buffer was used for these extractions to allow sufficient homogenisation of the lysis suspension following starch swelling
[3] DNA concentration was measured using the Picogreen® dsDNA quantitation kit [9]

3 Homogeneity

3.1 Homogeneity assessment

Due to the processing of pure non-GM and GM potato materials, the CRM powders are homogeneous with respect to the certified properties. Purity of the base materials has been tested and was reported before (see Section 2.1).

3.2 Minimum sample intake for analysis

As the CRMs consist of genetically pure materials, no minimum sample intake needs to be specified.

Rather, the sample intake is determined by the required specifications of the downstream use of the materials. Some guidelines for sample intake, based on experimental analysis, are given in section 2.4.3 and section 7.

4 Stability

4.1 Short-term stability

In order to assess whether special care must be taken during transportation, the short-term stability of dried potato powder was investigated. This was done for ERM-BF421a and it can be assumed that ERM-BF421b, produced and stored under the same conditions, behaves similarly.

During the short-term stability study, an isochronous approach [11] was applied and bottles, closed under argon, were exposed to 4 °C, 18 °C or 60 °C for 2 and 8 weeks. Genomic DNA was extracted from the CRMs using a CTAB procedure (Table 5; no α-amylase added). The DNA integrity of the samples was analysed by gel electrophoresis and the extractable DNA mass fraction was determined by dsDNA quantification using Picogreen® [9]. The results were compared to results obtained for samples stored at a reference temperature of -70 °C.

Figure 4 summarizes the results obtained during the study of 8 weeks. DNA quantification data confirmed that samples can be exposed for 8 weeks to temperatures of up to 18 °C and up to 2 weeks to temperatures of 60 °C without any DNA mass fraction loss. No significant DNA degradation was seen on the gels for any of these samples (Figure 5). Longer exposure to 60 °C (i.e. 8 weeks) reduced the extraction efficiency by 20 % and resulted in a slight increase in DNA degradation seen upon gel electrophoresis (Figure 5); moreover, a colour change of the dried powder to darker yellow/brown was observed for bottles exposed to 60 °C.

It is concluded that the potato CRMs can be dispatched under ambient conditions without an adverse effect on the stability of the materials.

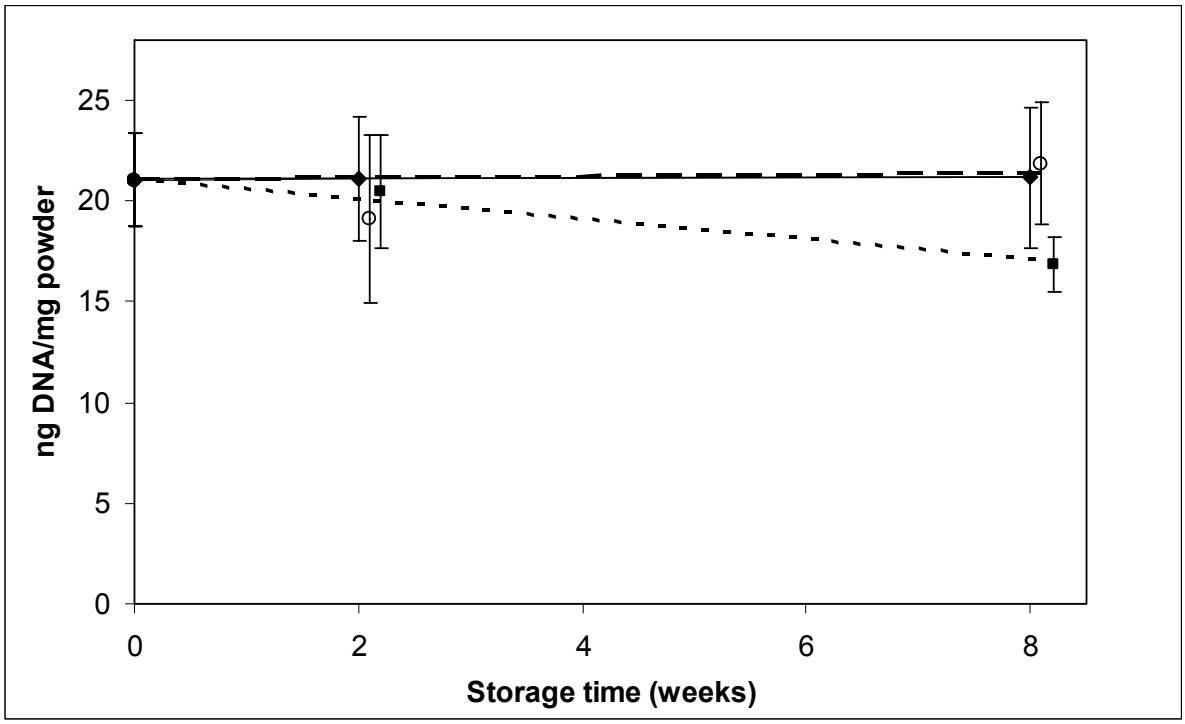


Figure 4: Short term-stability of ERM-BF421a at 4 °C (♦; ——), 18 °C (o; – – –) and 60 °C (■; - - -), measured by mass fraction of extractable DNA (determined by Picogreen dsDNA quantitation [9]). The error bars indicate *s* for *N* = 16.

Storage temp.:	-70 °C	4 °C		18 °C		60 °C		M
Time (weeks):	8	2	8	2	8	2	8	

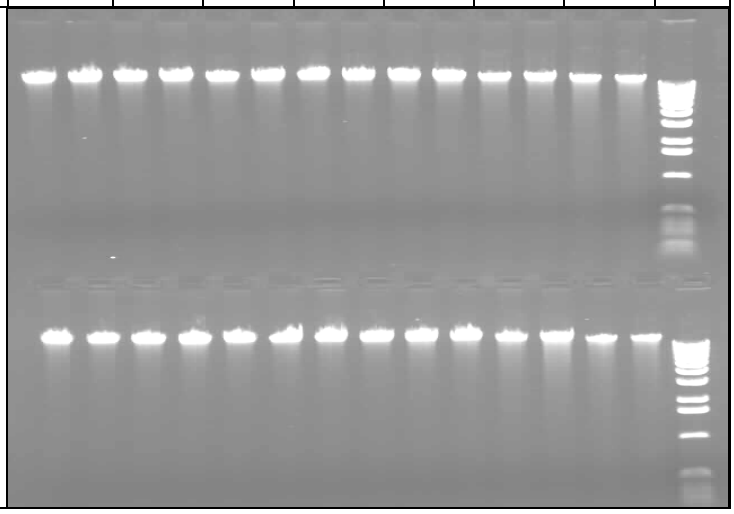


Figure 5: Gel electrophoresis of genomic DNA extracted from ERM-BF421a after exposure of the CRM to different temperatures during 2 or 8 weeks; four DNA extracts are shown for every time/temperature condition, 2 in the upper and 2 in the lower panel of the gel; M, 1 kb molecular DNA marker.

4.2 Long-term stability

A long-term stability study of ERM-BF421 covering 6 months at 4 °C or at 18 °C was organised. For ERM-BF421a, the analysis was done both after 3 months and after 6 months isochronous storage; only the 6 months data are reported here. A similar study was performed for ERM-BF421b, but only the 3 months data are currently available.

Monitoring of the stability of the potato CRM by gel electrophoresis indicated no instability at any of the temperatures investigated (Figure 6 and 7). For ERM-BF421b, also the EH92-527-1 potato copy number fraction (ratio between GM DNA copy number and the copy number of the endogenous reference gene) was determined by real-time PCR. The results Figure 8) revealed no significant difference between the different storage conditions (95 % confidence interval).

It is concluded that ERM-BF421 can be stored at 4 °C in the dark and under argon without any apparent effect on its stability.

Post-certification monitoring is being carried out at half-yearly intervals in order to keep monitoring the long-term stability of ERM-BF421.

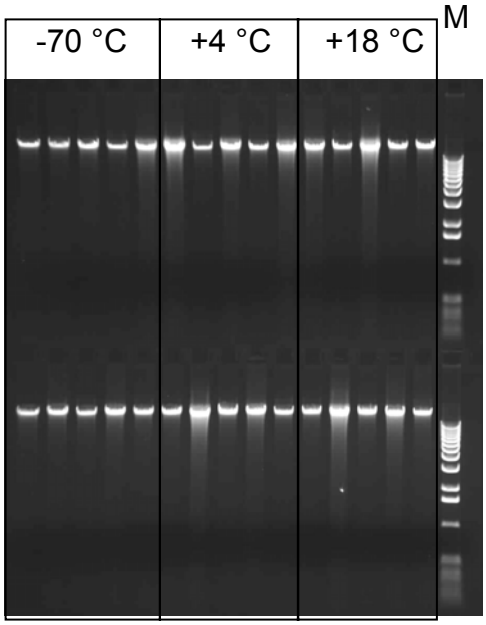


Figure 6: Stability of ERM-BF421a, stored during 6 months at -70 °C, 4 °C or 18 °C, visualised by gel electrophoresis (*N* = 5, *n* = 2 for each temperature condition); M, 1 kb DNA marker.

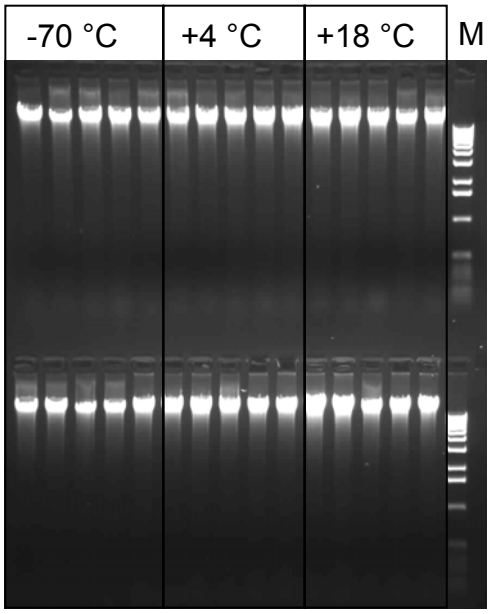


Figure 7: Stability of ERM-BF421b after storage during 3 months at -70 °C, 4 °C or 18 °C as visualised by gel electrophoresis (*N* = 5, *n* = 2 for each temperature condition); M, 1 kb DNA marker.

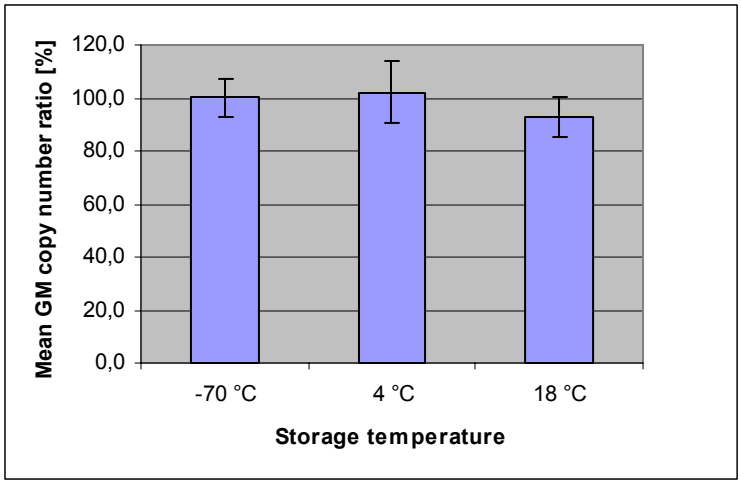


Figure 8: Stability of ERM-BF421b stored during 3 months at the temperatures indicated and assayed by real-time PCR analysis; mean GM copy number ratio [%] is shown with the error bars denoting the *s* (*N* = 5, *n* = 2, and rt-PCR in triplicate for each temperature condition).

5 Genetic identification of EH92-527-1 potato

The GM identity of both CRMs was verified using an event-specific real-time PCR method [5]. As an amplification control confirming the presence of amplifiable DNA, the potato-specific UGP reference gene was employed. The results, shown in Table 7, confirmed that ERM-BF421b contains the amplicon specific for EH92-527-1 potato. In contrast, ERM-BF421a, which gave a strong amplification signal for the UGP control gene (average Ct-value = 16.4) but no signal for the GM event, does not contain measurable material derived from EH92-527-1 potato.

Table 7: Identity confirmation of ERM-BF421 by event-specific real-time PCR

CRM	Genetic modification[1]	*N*	Copy number ratio $\overline{X} \pm s$ [%]
ERM-BF421a	Non-GM	12 [2]	< 0.2 [3]
ERM-BF421b	GM	20 [2]	101.1 ± 9.4 [4]

[1] GM refers to the EH92-527-1 modification
[2] For each CRM, genomic DNA was extracted by the CTAB method [10] from two subsamples from each of 6 or 10 randomly selected bottles for ERM-BF421a and ERM-BF421b respectively, and each DNA extract was analysed by rt-PCR in triplicate
[3] Each rt-PCR reaction contained 200 ng DNA and was run for 45 cycles. The measured values were below the LOD of the method being 0.2 %
[4] Each rt-PCR reaction contained 10 ng DNA and was run for 40 cycles

The junction region between the potato genome and the genetic insertion in EH92-527-1 was also sequenced by dideoxy chain termination methodology. The targeted region is expected to cover the junction between the potato genomic DNA and the promoter of the *Agrobacterium tumefaciens* nopaline synthase gene (NOS) that is part of the T-DNA insert in EH92-527-1. The 134 bp border sequence was amplified from genomic DNA purified from ERM-BF421b using the same flanking primers used in rt-PCR, then purified and sequenced from both ends with the PCR primers. The DNA sequence obtained by alignment of the seven overlapping partial sequences completely matched the amplicon sequence reported for this junction region by BASF Plant Science [5]. This confirms that ERM-BF421b contains material from EH92-527-1 potato.

6 Certified parameters and uncertainty budgets

6.1 Metrological traceability

ERM-BF421a and ERM-BF421b form a set of two reference materials certified for their number fraction of EH92-527-1 potatoes and for their genetic identity. This certification is based on the use of genetically pure (GM or non-GM) materials and the exclusion of any possible contamination between both materials during processing. Genetic purity of the materials was shown by qualitative assessment of the amylose content in every individual potato using a colorimetric staining method; this amylose method was validated by comparison to event-specific qualitative PCR, confirming that the absence of an amylose signal in GM potatoes coincided with the presence of the amplification product specific for the GM event EH92-527-1 (and vice versa for the non-GM potatoes). Event-specific real-time PCR on the CRMs confirmed that ERM-BF421b contained EH92-527-1 potato and ERM-BF421a contained no EH92-527-1 potato, consistent with previous data. The GM-specific amplification product was furthermore sequenced and the sequence was shown to be identical to the reported sequence around the GM insertion in EH92-527-1 potato [5].

6.2 Certified properties

The certified EH92-527-1 fraction is based on the number of EH92-527-1 and non-modified potato tubers used for the processing of both CRMs. EH92-527-1 potato tubers were individually identified through the amylose test, which was validated by analysing a subsample of potatoes by event-specific PCR, targeting the insert border region.

The genetic identity of ERM-BF421 was proven by sequence analysis of the border region between the T-DNA insert and the potato genome in EH92-527-1 potato, confirming the presence of the specific GM modification in EH92-527-1 potato as reported by the company and referring to the unique identifier code.

6.3 Uncertainty budgets

The exclusive use of genetically pure base materials and the absence of any mixing of ground materials, in combination with controlled production techniques, allow certifying the EH92-527-1 potato fraction in the CRMs with no uncertainty.

The nucleotide sequence obtained for the GM insertion event was based on the alignment of 7 overlapping partial sequences (obtained with forward and reverse primers) and allowed reconstitution of the complete 134 bp sequence without ambiguity.

7 Instructions for use

ERM-BF421 consists of two CRMs that are intended for use as reference materials for the detection of EH92-527-1 potato in food and feed. These CRMs are composed of milled, dried potato powder sealed in glass bottles under argon. The materials are hygroscopic and the customer is therefore advised to use the materials immediately after opening of the bottles.

The sample intake is determined by the planned use of the materials. If the materials are used for the extraction of genomic DNA, one should be aware that the composition of the potato powder, i.e. the starch constituents, differs between ERM-BF421a and ERM-BF421b and that this may affect their DNA extractability. Some experimental data and a detailed procedure for DNA extraction from these powders using the commonly employed CTAB method are given in Section 2.4.3 and the customer is advised to use this information as a guideline.

As a consequence of the observed differences in the mass fraction of extractable genomic DNA between both CRMs, the customer is strongly advised not to prepare gravimetric mixtures of both CRMs and to use the purified DNA from such mixtures for calibration purposes.

References and acknowledgements

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Authors: W. Broothaerts, A. Bau', J. Charoud-Got, M. Contreras, P. Corbisier, P. de Vos, H. Emteborg, N. Meeus, A. Oostra, K. Teipel, S. Trapmann, H. Emons
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Abstract

This report describes the preparation and certification of the potato powder Certified Reference Materials (CRMs) ERM-BF421a and ERM-BF421b, consisting of conventional and genetically modified EH92-527-1 potato powder, respectively.

The CRMs were processed in 2005/2006 and were certified in 2006 by the European Commission, Directorate General Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium.

The EH92-527-1 genetic modification results in tubers containing nearly exclusively amylopectin (> 98 %) as the starch component, whereas conventional potatoes have starch composed of both amylose and amylopectin in a ratio of 1:3.

For the processing of the CRMs, tubers of non-modified potato and EH92-527-1 potato, supplied by BASF Plant Science GmbH (Ludwigshafen, DE), were individually tested for the presence of the genetic modification, then brushed under tap water, cut into cubes and freeze-dried. After grinding, a pure non-modified potato powder and a pure EH92-527-1 GM potato powder were obtained. The certification of the CRMs was based on the individual testing of the potato tubers used for the production of the CRMs, using an amylose test as a measure for the presence of the genetic modification, and was furthermore confirmed by real-time PCR analysis of the CRMs. The identity of the EH92-527-1 specific modification was also confirmed by nucleotide sequence analysis of the junction region between the plant DNA and the genetic insertion.

The CRMs are intended to be used as positive and negative controls for the detection of genetically modified EH92-527-1 potato in food and feed. The CRMs are available in glass bottles containing 1 g (ERM-BF421a) or 0.5 g (ERM-BF421b) of potato powder, closed under argon atmosphere.

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