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Development of a Certified Reference Material for Genetically Modified Potato with Altered Starch Composition

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The presence of genetically modified organisms (GMOs) in food and feed products is subject to regulation in the European Union (EU) and elsewhere. As part of the EU authorization procedure for GMOs intended for food and feed use, reference materials must be produced for the quality control of measurements to quantify the GMOs. Certified reference materials (CRMs) are available for a range of herbicide- and insect-resistant genetically modified crops such as corn, soybean, and cotton. Here the development of the first CRM for a GMO that differs from its non-GMO counterpart in a major compositional constituent, that is, starch, is described. It is shown that the modification of the starch composition of potato (*Solanum tuberosum* L.) tubers, together with other characteristics of the delivered materials, have important consequences for the certification strategy. Moreover, the processing and characterization of the EH92-527-1 potato material required both new and modified procedures, different from those used routinely for CRMs produced from genetically modified seeds.

KEYWORDS: Amylopectin; amylose; Certified Reference Material; CRM; DNA extraction; genetically modified organism; GMO; potato; real-time PCR; *Solanum tuberosum* L.; starch

INTRODUCTION

To inform the consumer, legislation in the European Union (EU) demands the labeling of food and feed products containing >0.9% of genetically modified organisms (GMOs), calculated per ingredient (*1*). Different and currently still debated thresholds have been suggested for seeds and, in principle, a zero tolerance has been set for GMOs that are not authorized in the EU. The GMO regulatory compliance in Europe, and similarly in other countries, necessitates on the one hand development of reliable quantification methods for the respective GMOs and, on the other hand, the provision of reference materials to control the correct application of the methods. In practice, a GMO producer needs to provide a quantification method for the GMO to the Community Reference Laboratory (CRL) for GM Food and Feed, which arranges its technical evaluation and validation. Additionally, the company has to indicate where the reference material is available and therefore may request the production of a GMO reference material to a reference material producer, such as the European Commission's Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Accessibility to GMO reference materials indeed forms a prerequisite before the European Food Safety Authority (EFSA, Parma, Italy) will issue an overall opinion on the safety of the GMO for humans and for the environment. A positive risk assessment, successful validation of the quantification method, and accessibility to a reference material form the prerequisites before a GMO or any food and feed consisting of, containing, or produced from the GMO in question may be granted approval to enter the European market.

Reference materials for genetically modified (GM) plants are commonly available as matrix Certified Reference Materials (matrix CRMs) (*2*, *3*). They are processed from seeds of the GM line and a comparable non-GM variety. The resulting products are homogeneous powders, which are often supplied at different GMO mass fractions, typically about 0, 0.1, 1, and 10% (m/m) (see http://www.irmm.jrc.be/html/reference_materials_catalogue/index.htm). Genomic DNA can be extracted from these materials for the in-house validation of quantification methods, for use as a calibrant in quantitative measurements or for quality control purposes.

During the processing of the CRMs, the GMO mass fraction in the gravimetric mixtures is verified by performing real-time PCR, the common method for quantification of GMOs (*4*). Real-time PCR measures the abundance of DNA sequences specific for the GMO in relation to the abundance of DNA of a species-specific reference gene, yielding a DNA copy number ratio for the material being analyzed. As the CRMs themselves are certified for their GMO mass fraction, the assumption is implied that there exists a conserved proportionality between the mass of an ingredient and the total number of genes or genomes contained in it. In reality, several sources of variability may lead to distorted mass versus gene dosage ratios (*5*). Addressing this issue, the EC has proposed to define the 0.9% threshold

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for labeling as a GMO copy number percentage instead of the current implicit mass percentage (*6*).

The list of CRMs developed for GMO detection currently includes those for maize, cotton, rapeseed, and soybean lines modified for insect and/or herbicide resistance (see http://ec.europa.eu/food/food/biotechnology/authorisation/index_en.htm). The genetic modification in these GMOs resulted in the expression of one or at most a few heterologous proteins without changing the plant's cell structure or overall composition. Theoretically, and from the concept of certifying GMO mass fractions, the processing of such GMO and non-GMO seeds into a CRM was rather straightforward because both raw materials were substantially equivalent. In contrast, we describe here the processing and certification approach for a nonseed material of which the GMO raw material was essentially different from its non-GMO counterpart. The EH92-527-1 potato line is a processing potato variety, developed to produce and store an abundance of starch granules in the mature tubers. Conventional starch potatoes produce a 1:5 mixture of the linear (α-1,4) glucose polymer amylose and its (α-1,6) branched form amylopectin, the common constituents of starch. In contrast, as a result of the inhibition of the granule-bound starch synthase (*gbss*) gene, the EH92-527-1 GM potatoes synthesize nearly exclusively amylopectin with little or no amylose remaining in the starch granules (*7*). The altered composition of the GMO versus non-GMO vegetative materials necessitated a new design of the certification strategy for this first nonseed GMO CRM, named ERM-BF421.

MATERIALS AND METHODS

Plant Materials. *Solanum tuberosum* L. tubers from the 2004 harvest of the non-GM starch potato variety Kuras and the GM starch potato event EH92-527-1 were received from Plant Science Sweden, a BASF Plant Science subsidiary. Quality control by the delivering company through real-time PCR revealed that the genetic modification was absent from 50 randomly selected tubers of the non-GM batch, and that it was present in 50 randomly selected tubers of the GMO batch, resulting in a statistical purity of at least 94% (95% confidence level). The tubers were stored well-aerated in cardboard boxes at a constant temperature of 7 °C in the dark. Approximately 75 kg of conventional and 35 kg of GM potatoes were used for the processing.

Amylose Assay. A surface chip (1−2 cm^2) was cut from each potato and stained for amylose by applying Lugol's solution (*8*) onto the cut surface of the chip. A color change to dark blue, scored after 1 min, indicated the presence of amylose, whereas an unchanged orange/brown color of the iodine-containing staining solution marked the absence of significant amylose levels. The reliability of this test was verified by comparison with event-specific PCR beforehand.

Processing. Cross-contamination and contamination with foreign DNA were avoided using glove box systems and treatment of all contact surfaces with DNAerase (MP Biomedicals, Irvine, CA), 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. The tubers were brushed and rinsed under tap water to remove the remaining soil and left to air-dry. Following amylose-testing and the removal of deteriorated parts, the tubers were cut into fries with a manual potato chipper and further into cubes of about 1 cm^3. Freeze-drying for 133 h of primary drying and 2−10 h of secondary drying resulted in a two-thirds 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 amber glass vials. Filling was done under ambient temperature conditions using an automatic filling machine contained in a glove box with argon atmosphere. The filling mass (1 and 0.5 g for non-GM and GM materials, respectively) was checked at regular intervals on a calibrated external balance. To maintain the inert atmosphere, a nozzle flushed the vials with argon prior to filling, and rubber stoppers were pressed into the neck of the vials after filling. Prior to capping, an acousto-optical tunable near-infrared spectrometer (AOTF-NIR; Brimrose, Baltimore, MD) recorded a NIR spectrum from every vial. The vials were capped using differently colored aluminum caps for the non-GM and GM powders and labeled using an automatic assembly line respecting the filling order. Following inventorization, the vials were stored at a constant temperature of 4 °C in the dark.

Water Determination and Particle Size Analysis. Water determination was based on AOTF-NIR measurements on the individual vials, calibrated with a meat powder containing water mass fractions of 5−85 g/kg (*9*). A number of randomly selected vials were also directly analyzed by volumetric Karl Fischer titration (KFT) on a 758 KFD Titrino (Metrohm AG, Herisau AR, Switzerland).

Micrographs were taken from the powders using a Zeiss microscope Stemi 2000-C (Oberkochen, Germany). Particle size distribution was verified by sieve analysis on an Alpine Sieve Analyzer 200LS-N (Hosakawa Micron Group, Osaka, Japan) following ISO 3310-1 (*10*) 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 vials (10 for the non-GM and 20 for the GM powder) were merged to reach the required sample intake of 10 g for sieving analysis. Particle sizes were also measured by laser diffraction using a Helos particle size analyzer (PSA, Sympatec, Clausthal-Zellerfeld, Germany). The instrument measures particle size distribution in terms of the equivalent sphere diameter in the range 0.5−875 μm. For both CRMs, three replicate samples from each of five randomly selected vials were analyzed. It was noted that 1 of the 15 subsamples from both CRMs showed the presence of coarse particles exceeding the measuring range. From the sieve analysis, it was found that the mass contribution of these coarse particles to the total powder mass was negligible; therefore, the results for these subsamples were omitted from the calculation of the average particle size distribution of both CRMs.

Determination of the DNA Mass Fraction in Both CRMs. To verify the total DNA mass fraction in both powders, a slight modification of the fractionation method developed initially by Ogur and Rosen was employed (*11*). Following the sequential removal of ethanol-, ethanol-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 derivatization with diphenylamine. Diphenylamine reacts specifically with 2-deoxyriboses linked to purine nucleobases (*12*). The method was validated in-house by quantification of the recovery of spiked lambda DNA and calf thymus DNA into potato powder. For each potato material, nine replicates of 1 g of powder were analyzed, and the results were statistically evaluated using one-way ANOVA.

The DNA mass fraction in both CRMs was also assessed using a modified CTAB extraction method (*13*), optimized for the extraction of DNA from potato powder. One milliliter of hot (65 °C) CTAB extraction buffer [1.4% (m/v) CTAB, 1 M NaCl, 0.1 M Tris-HCl, 0.15 M Na_2EDTA, pH 8.0] was mixed with 150 mg (GM) or 200 mg (non-GM) of potato powder. Ten microliters of RNase A (100 mg/mL) and 20 μL of proteinase K (20 mg/mL) were added, and the microtubes were incubated for 30 min at 65 °C in a thermomixer with 1400 rpm. Following centrifugation (10 min, 13000 rpm), the supernatant was decanted into a new tube containing 500 μL of chloroform, shaken, and centrifuged (10 min, 13000 rpm) to separate the phases. The organic extraction was repeated once more with an equal volume of chloroform. The upper phase was then mixed with 2.2 volumes of CTAB precipitation buffer [0.5% (m/v) CTAB, 40 mM NaCl, 50 mM Tris-HCl, pH 8.0] and left at room temperature for 1 h. The DNA pellet was precipitated by centrifugation (10 min, 13000 rpm), redissolved in 400 μL of 1.2 M NaCl, and re-extracted with an equal volume of chloroform. Following centrifugation, the upper phase was mixed with 2 volumes cold (−20 °C) ethanol, and the tubes were incubated at −20 °C (for at least 10 min) and then centrifuged for 10 min at 13000 rpm. The DNA pellet was finally washed with 70% cold EtOH and

Table 1. Oligonucleotides Used in This Study (See http://gmo-crl.jrc.it/statusofdoss.htm)

target	oligo	nucleotide sequence	amplicon length (bp)
ugp	forward	5′-GGA CAT GTG AAG AGA CGG AGC-3′	88
	reverse	5′-CCT ACC TCT ACC CCT CCG C-3′	
	probe	5′-(FAM) CTA CCA CCA TTA CCT CGC ACC TCC TCA (TAMRA)-3′	
event527	forward	5′-GTG TCA AAA CAC AAT TTA CAG CA-3′	134
	reverse	5′-TCC CTT AAT TCT CCG CTC ATG A-3′	
	probe	5′-(FAM) AGA TTG TCG TTT CCC GCC TTC AGT T (TAMRA)-3′	

resuspended in 50 or 100 μL of nuclease-free water. To ensure complete resuspension, the DNA solution was left overnight at 4 °C and then incubated for 30 min at 37 °C under agitation before use. To improve the DNA extraction, 1 μL of the heat-stable α-amylase from *Bacillus licheniformis* (Sigma, catalog no. A3403) was added during the lysis step to degrade the starch. The amount of extracted DNA was determined by fluorometry on a FluoStar Galaxy microplate reader (BMG Labtech, Offenburg, Germany) using the Picogreen dsDNA quantitation kit (Molecular Probes Inc., Eugene, OR). Statistical differences between data sets were analyzed using the *F* test and the *t* test.

Qualitative and Real-Time PCR Analysis. Genomic DNA was extracted from freeze-dried potato powder according to a modified CTAB method. Qualitative PCR was performed in a total reaction volume of 25 μL with 12 ng of genomic DNA and containing 1× PCR reaction buffer (Invitrogen, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 300 nM primers for Event527 (targeting the sequence junction between the plant and the GM insertion in EH92-527-1) or 400 nM primers for *ugp* (**Table 1**), and 0.625 unit of Platinum Taq DNA polymerase (Invitrogen). Amplification was done in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA), starting with a 2 min denaturation at 94 °C, then 35 cycles of 15 s at 94 °C and 60 s at 60 °C, and a final 1 min extension step at 72 °C. The complete reaction solution was mixed with 2.5 μL of 10× loading dye (Invitrogen) and run through a 2% (m/v) agarose gel, which was stained with ethidium bromide.

Real-time PCR was performed in an ABI 7900HT apparatus (Applied Biosystems) in 96-well format. Each reaction was run in triplicate and contained 1× TaqMan Universal PCR Mastermix (Applied Biosystems) and oligonucleotides (300 nM primers and 160 nM probe for Event527 or 400 nM primers with 200 nM probe for *ugp*) in a total volume of 25 μL. GM quantification was based on calibration curves for *ugp* and for Event527, prepared by diluting EH92-527-1 genomic DNA in nuclease-free water (Promega, Madison, WI). The GM percent was determined as the ratio between the absolute number of GM copies relative to the number of copies of the *ugp* reference gene. For analysis of ERM-BF421a (non-GM potato powder), each PCR reaction contained 200 ng of DNA and was run for 45 cycles. For ERM-BF421b (GM potato powder), each reaction contained 10 ng of DNA and was run for 40 cycles. The average PCR efficiencies obtained were 102 and 100% for *ugp* and Event527, respectively, and a good linearity (R^2 of >0.99) was found for all calibration curves. The LOD of the method was taken as the mean LOD determined from four independent calibration curves prepared from genomic DNA extracted on four different days and was calculated as 3.3 times the standard deviation of the lowest concentration tested, multiplied with this concentration.

Nucleotide Sequence Analysis and BLAST Analysis. Genomic DNA was isolated from ERM-BF421b ($N = 2$), amplified with event-specific primers in a volume of 50 μL, purified twice on a Qiaquick PCR purification column (Qiagen, Hilden, Germany), and directly sequenced from both sides by dye terminator cycle sequencing on a CEQ8000 Genetic Analysis System (Beckmann Coulter Inc., Fullerton, CA) using the GenomeLab Methods Development Kit (Beckmann Coulter Inc.). The seven sequences obtained were manually aligned and compared to the amplicon sequence confidentially obtained from BASF Plant Science. A BLAST search was performed on the whole sequence using BLASTN 2.2.15.

RESULTS AND DISCUSSION

Development of the Certification Strategy. The IRMM was requested to develop and produce a CRM for the GM EH92-527-1 potato, an amylopectin-type starch potato. The Institute is accredited for producing Reference Materials according to ISO Guide 34:2000 (*14*). CRM production includes as one of the first steps the analysis of the purity of the delivered starting materials. For seed lots, the purity can be realistically assayed only by investigating a certain number of subsamples obtained according to a sampling strategy and extrapolating the resulting data to the purity of the whole batch. For a material consisting of potato tubers, the number of individual biological entities required for the processing was obviously much lower. This provided the opportunity for purity testing of each tuber, thus reducing the uncertainty on the certified values. Additionally, any impurity of the potato materials was considered to be less likely for this vegetatively propagated crop compared to a hybrid seed lot resulting from cross-pollination.

For commutability with real samples, most GMO CRMs are matrix reference materials with the concentration of the measurand of interest, that is, the GMO, in accordance with the measurand concentration in the samples to be routinely analyzed by the laboratories (for further reading on the terms used, see ref *3*). The EH92-527-1 potato is, according to the application for authorization, primarily intended for starch processing into industrial products and use of the byproducts in animal feed (*15*). The samples for laboratory analysis will therefore consist of either whole potatoes collected during transportation or feed and food. Although the former can be qualitatively assessed with the help of quality control materials (GMO and non-GMO), quality assurance for the analysis of food and feed samples stipulates the preparation of CRMs containing defined EH92-527-1 fractions. There existed, however, a principal objection against the preparation of gravimetric mixtures of GMO and non-GMO materials for this GM potato. Provided that GMO quantification is targeting DNA sequences, the mixing of two materials can usually be performed only if the number of DNA copies per mass unit is the same in both starting materials. There were several arguments to doubt if this was really the case here. The genetic modification itself, shifting the starch biosynthesis away from amylose, resulted in an altered starch composition in the GMO tubers, and starch constitutes 70% of the dry mass of a tuber. Despite the reporting of a conserved total starch mass (*7*), a decreased dry matter content has been measured for the EH92-527-1 potatoes compared to the parental comparator variety during a 3 year field trial (*15*). As a consequence, the DNA/mass ratio may be different in the GM potatoes compared to that in the non-GM tubers. Furthermore, the visual inspection of the delivered materials showed that the GM potatoes were significantly smaller that their non-GM comparators and thus have relatively more peel (**Figure 1a**). The cells constituting the peel layer are much smaller in size than the starch-stuffed cells of the flesh of the tuber, and smaller cells means more cells for the same mass and, hence, more DNA per mass. Consequently, the increased peel mass fraction in the smaller GM potatoes may also influence the DNA/mass ratio. Finally, feasibility studies at IRMM showed that the DNA extractability differed significantly between the GM and non-GM potatoes

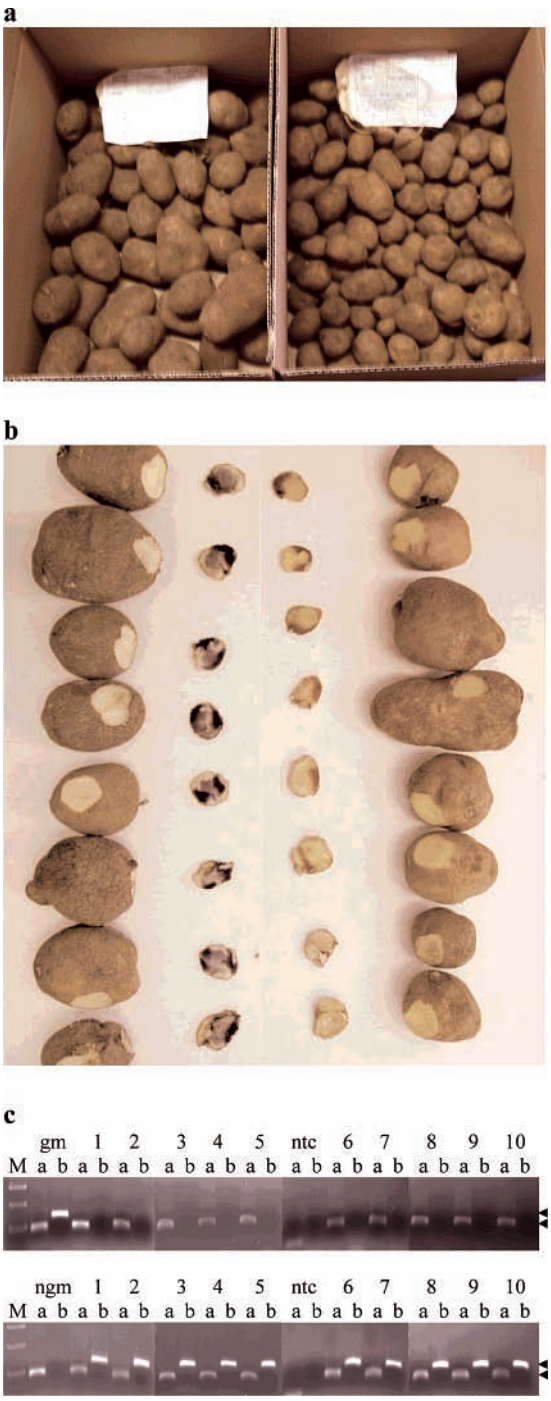

Figure 1. Delivered raw potato materials and purity analysis of the batches. (**a**) non-GM (left) and GM (right) potato sub-batch; (**b**) amylose testing of individual non-GM (left) and GM potatoes (right) (blue coloration in the non-GM potatoes indicates the presence of amylose); (**c**) qualitative PCR on 10 non-GM (upper panel) and 10 GM potatoes (lower panel) for the potato *ugp* reference gene (lane a, lower arrowhead) and the GM event (lane b, upper arrowhead). GM (gm) and non-GM (ngm) DNA was used as control DNA during the testing of non-GM and GM potatoes, respectively. ntc, no template (negative) control; M, 100, 200, and 300 bp DNA marker fragments.

(see below), leading to more DNA extracted from the non-GM compared to the GM powder. Under these circumstances, it could not be guaranteed that both potato materials had similar DNA contents and, hence, their metrologically sound mixing could not be substantiated. Instead, separate "pure" matrix CRM were produced for each of the two raw materials.

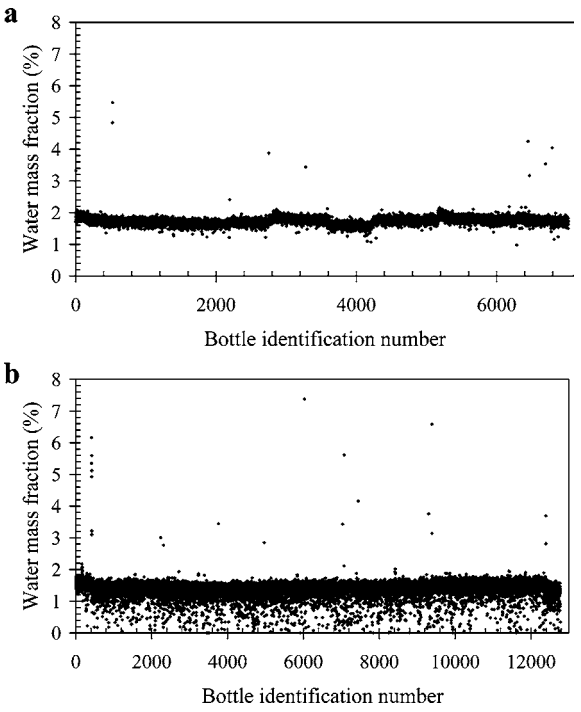

Figure 2. Water mass fraction determined by AOTF-NIR spectrometry during filling of the non-GM CRM (**a**) and the GM CRM vials (**b**).

Purity Assessment and Confirmation of Genetic Identity. The delivered raw materials consisted of potato tubers of the conventional starch potato variety Kuras and the GM variety EH92-527-1, derived from the parental line Prevalent through *Agrobacterium*-mediated transformation (**Figure 1a**). For individual testing of a total of >1000 tubers needed for the processing, a quick, qualitative method was adopted providing an indirect measurement of the genetic modification. A superficial chip was cut from each tuber and stained for the presence of amylose by reaction with iodine (**Figure 1b**). The 549 non-GM tubers tested scored positive for amylose in this test, whereas the 493 GM tubers were negative, confirming their impaired amylose biosynthetic capacity. Both materials were also visually identified by the color of the cut tuber flesh, which was whitish and yellow for the non-GM and GM tubers, respectively (**Figure 1b**).

To ensure that the absence of amylose in the GM potatoes resulted from the specific EH92-527-1 insertion, we extracted genomic DNA from 10 randomly selected GM tubers and performed PCR with event-specific primers encompassing the 5′ T-DNA insertion site (**Table 1**). The same approach was applied to 10 non-GM tubers, and the potato *ugp* gene was used as an amplification control for all DNA extracts. The results, shown in **Figure 1c**, revealed the presence of the EH92-527-1 event in the amylose-negative GM tubers only. As a final proof of GM identity, the nucleotide sequence of the amplified GM fragment was determined. The sequence obtained matched the 134 bp amplicon sequence reported for this junction region by BASF Plant Science (data not shown), confirming that the EH92-527-1 event is present in the delivered GM materials. Upon BLAST analysis, the amplicon sequence revealed similarity only in its 3′ half to the T-DNA region immediately adjacent to the right border in many binary vectors, substantiating the expectation that it covers the junction region between the plant DNA and the T-DNA insertion in the EH92-527-1 potato.

Processing. To prevent accidental contamination, the GM and non-GM raw materials were processed separately, starting with the non-GM material. Whole tubers were used for the

Table 2. DNA Mass Fraction in Non-GM and GM Potato

DNA extraction method	DNA mass fraction (μg of DNA/g of potato powder) non-GM potato	GM potato
fractionation + derivatization	107 ± 17 (9)[a]	123 ± 16 (9)
CTAB – α-amylase	20.6 ± 3.1 (67)	9.5 ± 2.6 (12)[b]
CTAB + α-amylase	31.2 ± 2.6 (15)	23.1 ± 8.4 (60)

[a] Mean + *s*; number of extractions (*n*) given in parentheses. [b] Deviating from the standard protocol, the volume of CTAB extraction buffer was increased to 1.2 mL to allow sufficient homogenization of the lysis suspension despite excessive starch swelling.

processing because the starch-processing industry also uses unpeeled potatoes for the extraction of starch. Furthermore, preliminary studies showed that the peel contained significantly more DNA than the potato flesh (approximately 4 times more per dry mass). Following cutting, freeze-drying, and milling in a cryogenic mill, potato powders were obtained containing <20 g/kg remaining water, determined by volumetric Karl Fischer titration (KFT). Because the GM base material visually contained some larger particles (derived from the peel), the powder was sieved through a stainless steel sieve with a mesh of 0.5 mm. The mass of the sieved-out particle fraction was negligible compared to the total mass of the powder (2 g/kg), and it was, therefore, decided to discard it. Similar, but fewer, coarse particles were observed in the non-GM powder, but these were not sieved out. Microscopy and preliminary particle size analysis (PSA) confirmed that the particle size of the powders was adequate for further processing (see below).

During water determination in the bulk powders, the strong hygroscopy of the powders was noted. To prevent excessive and unequal water uptake during filling of the CRM vials, an automatic filling line was set up in a closed glove box filled with argon. The inert atmosphere was maintained until closure of the vials. The mass of potato powder filled into each of the vials was a minimum of 1 or 0.5 g for the non-GMO or GMO CRM, respectively. Monitoring of the water content in each of the vials prior to capping was accomplished by recording NIR spectra using a spectrometer (AOTF-NIR) integrated on-line into the filling instrumentation. The NIR spectra were subsequently evaluated using a PLS1 regression model based on meat calibrants with known water mass fractions, which allowed the prediction of the water content in each vial. The meat calibrants were previously shown to generate water mass fraction data for various matrices that corresponded well with data determined by KFT (*9*). The NIR data obtained for both potato powders showed that the water mass fraction (mean ± *s*) was 17.4 ± 1.5 g/kg for the non-GMO powder and 13.5 ± 3.5 g/kg for the GMO powder (**Figure 2**). Measuring within a spot of 6 mm in diameter, some spurious data were occasionally obtained when the filled powder was not attached to the inner wall of the vials at the measurement spot. The higher incidence of scattering in the GMO powder resulted from the smaller powder mass filled into the vials. The observed low water mass fractions in both powders remained constant over the fill series, indicating that water was not accumulating during filling (**Figure 2**). The results were also consistent with KFT measurements on the same powders.

The resulting powders were carefully checked for their particle size distribution both by sieve analysis and by laser diffraction measurements. The results indicated that >50% of particles had a size of <45 μm, and the maximum size of particles observed was below 515 μm. A similar, unimodal size distribution pattern was obtained for both the GMO and non-GMO powders. The particle characteristics were considered as appropriate for an efficient extraction of genomic DNA (*16*).

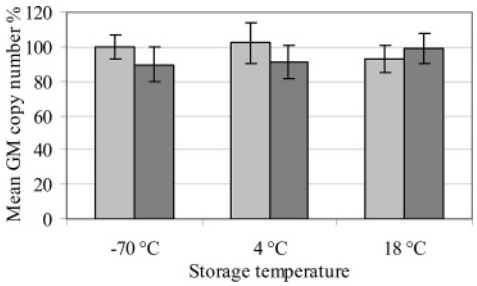

Figure 3. Long-term stability of GM potato CRM evidenced by quantification of the GM copy number percent by real-time PCR following isochronous incubation at the indicated temperatures during 3 or 6 months.

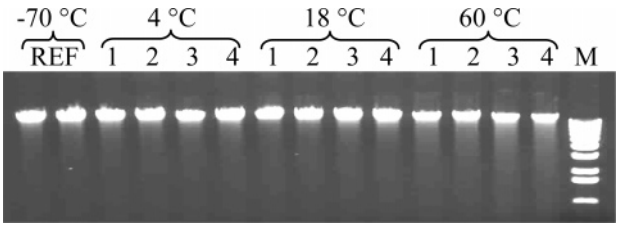

Figure 4. Stability of potato CRM during short-term isochronous incubation at the indicated temperatures for 2 weeks (lanes 1 and 2) or 8 weeks (lanes 3 and 4) in comparison with incubation at the reference temperature (REF). Agarose gel electrophoresis of genomic DNA extracted from non-GM potato CRM was performed; two DNA extracts are shown per test condition. M, 1–12 kb DNA markers.

DNA Content. As a consequence of the intrinsic qualities of the GMO and non-GMO powders, we hypothesized earlier that their DNA content per powder mass may not be equal. To provide further evidence for this, their total DNA mass fractions were investigated by destructive spectrophotometric analysis of nucleotide derivatives following chemical fractionation. The results are shown in **Table 2**. Statistical analysis confirmed that there was a significant difference in total DNA mass fraction between the GMO and non-GMO powders (95% confidence level). This result was consistent with the inclusion of a larger fraction of (DNA-rich) peel material into the GMO powder compared to the non-GMO powder (as discussed above).

To develop recommendations for the CRM user, the extractable DNA mass fraction in both powders was determined using a common laboratory extraction method involving CTAB (*13*). The CTAB method had to be modified to be applicable to the extraction of a reasonable amount of intact DNA from the potato matrix. As a result of the abundance of starch in the potato powders, cell lysis at 65 °C resulted in the swelling of the starch granules and formation of a viscous gelatinous suspension entrapping part of the DNA molecules. Although potato starch already has a very high swelling power, the swelling effect was even more pronounced in the EH92-527-1 powder as a result of the nearly exclusively branched amylopectin polymers constituting the modified starch. Consequently, the sample intake was reduced for the GMO powder (150 mg) compared to the non-GMO powder (200 mg) in order to allow an adequate mixing of the viscous suspension during lysis. Even under these conditions, the mass of extracted DNA from the GMO powder was less than half of that obtained from the non-GMO powder under the same conditions (**Table 2**). Attempting to improve

Table 3. Certification of ERM-BF421

CRM	ERM-BF421a		ERM-BF421b	
matrix	dried potato powder		dried potato powder	
certified property	number fraction[a] (%)	sequence identity	number fraction[a] (%)	sequence identity
certified parameter	EH92-527-1 potatoes	Event527 amplicon[b]	EH92-527-1 potatoes	Event527 amplicon[b]
certified value	0[c]	sequence absent[d]	100[c]	sequence present[e]

[a] Number of EH92-527-1 potatoes per total number of potatoes used in the processing. [b] DNA amplicon obtained by conventional PCR amplification with forward and reverse primers for Event527 (see **Table 1**). [c] Based on the colorimetric analysis of the amylose content in every individual potato used for processing and confirmation by event-specific real-time PCR. The uncertainty is considered to be negligible. [d] Based on event-specific real-time PCR analysis. The uncertainty is negligible. [e] Based on nucleotide sequence analysis and event-specific real-time PCR. The uncertainty is negligible.

the DNA extraction from the GMO powder, the enzymatic breakdown of the potato starch was investigated. During industrial starch processing, α-amylases are often added for starch modification during the liquefaction process. The α-amylase from *Bacillus licheniformis* was reported to efficiently degrade potato amylopectin starch (*17*); moreover, it is a heat-stable enzyme. Preliminary studies showed that the *Bacillus* α-amylase was active in CTAB extraction buffer at 65 °C. The addition of 1 μL of this α-amylase was sufficient to degrade most of the starch in the GMO potato lysis suspension and prevent gelation. Starch degradation strongly improved the DNA extraction from the GMO potato powder (**Table 2**). The same approach also increased the DNA recovery from the non-GMO powder. As a result, the enzymatic starch breakdown during DNA extraction with the CTAB method reduced but did not eliminate the different extractabilities of both powders (note that all CTAB data in **Table 2** are significantly different from one another at the 95% confidence level). It can be concluded from these results that any mixture of the powders on a gravimetric basis will not reflect equal DNA copy ratios and should therefore be avoided.

As the processed powders consisted of pure, unmixed materials, the minimum sample intake for preventing inhomogeneity is determined solely by the ability to extract a sufficient amount of DNA for downstream applications, that is, PCR. A 100 mg sample mass, as suggested in common laboratory extraction methods, largely fulfills this requirement for both powders.

Stability Studies. To determine the optimal storage conditions for these new materials, their stability was studied by isochronous incubation (*18*) at 4 and 18 °C during 6 months, with an intermediate evaluation after 3 months of storage. These samples were compared to samples kept at a reference temperature of −70 °C, assuming that the materials were stable at this temperature. Genomic DNA was extracted from the samples ($N = 5$, $n = 2$; see Abbreviations Used) and analyzed by gel electrophoresis. No sign of DNA degradation was apparent for any of the temperature conditions, and the DNA yields were similar for all samples analyzed (data not shown). For the GMO material, the storage stability was furthermore investigated by real-time PCR, revealing no significant difference in relative GM DNA copy numbers between the different incubation temperatures (**Figure 3**). For convenience, it was decided to store the potato reference materials at 4 °C and to continue assessing the stability every 6 months as part of the postcertification stability monitoring plan organized for all released CRMs of the IRMM.

To analyze the stability of the potato matrix during dispatch to the customers, a short-term isochronous study at 4, 18, and 60 °C was organized. Arguing that temperature would affect both dry potato matrices equally, the study was carried out on one of the powders only. Following 2 or 8 weeks of incubation at the defined temperatures or at −70 °C for reference, genomic DNA was extracted and subjected to gel electrophoresis ($N = 8$, $n = 2$). The results, shown in **Figure 4**, confirmed the stability of dried potato powder upon storage at the temperatures indicated. However, a slight DNA degradation after 8 weeks of incubation at 60 °C was noted, which also coincided with a color change of the powder from yellow to amber. Nevertheless, the results confirmed that the CRMs can be dispatched under ambient temperatures without any adverse effects on the stability of the materials.

Certified Parameters, Uncertainties, and Metrological Traceability. In the absence of any mixing, the two potato CRMs described here were direct processing products from the delivered raw potato tubers; every possible precaution was taken to avoid contamination during storage, processing, or filling. As a result of the individual purity assessment of all the tubers used for the processing, it has been proven beyond reasonable doubt that no EH92-527-1 potato has entered the non-GMO CRM and that all potato tubers used for processing into the GMO CRM were EH92-527-1 potatoes. This was furthermore confirmed by real-time PCR analysis revealing a GM copy number ratio that was either below the LOD of the method (0.1% m/m) or close to 100% [95.6 ± 10.1 (mean ± s) copy number %] for the non-GMO ($N = 6$, $n = 2$) and GMO CRM ($N = 28$, $n = 2$), respectively. The CRMs were, therefore, certified for the number fraction of EH92-527-1 potatoes, and the associated uncertainty on these values was considered to be negligible (**Table 3**). Metrological traceability of the certified values is based on the qualitative amylose staining test providing an indirect measure for the genetic modification, which was validated by comparison to GM event-specific PCR. The latter method detected the presence of the GM amplicon, specific for the EH92-527-1 variety, and the nucleotide sequence of this amplicon was confirmed by sequence analysis. As a result of the unbroken chain of comparisons, the CRMs could also be certified for their EH92-527-1 identity, tracing back to the Unique Identifier BPS-25271-9 specific for this GMO event (*13*). The CRMs were, principally, not certified for their EH92-527-1 mass fraction, consistent with the reasoning followed to reject the preparation of gravimetric mixtures between them. It would falsely give the customer the impression that a pure EH92-527-1 potato powder would contain the same mass of EH92-527-1 DNA per gram of powder as a pure non-GM potato powder. Consequently, mixing the two CRM materials at the customer's premises cannot provide the desired fraction of EH92-527-1 DNA copy numbers. As clearly stated before, the mixing approach cannot be supported for these materials. Certification of these CRMs for their GMO DNA copy number fraction (*6*) and expression of the GMO quantification results

in copy number ratios could resolve the problem of unequal DNA/mass ratios.

In conclusion, the development of a new set of CRMs is described here, which involved a number of scientific and technical challenges related to the type of materials processed and the specific genetic modification contained in one of them. The approach employed targeted the production of two pure matrix CRMs certified for the number fraction and identity of EH92-527-1 potatoes. This resulted from the individual confirmation of the tubers' identity and took into account that GMO and non-GMO powders may have contained different DNA fractions. Furthermore, the issue of DNA extractability of the starch-containing materials was investigated and the effect of the starch composition discussed. This certification also highlights the need for harmonization of what is understood as "GM %" with regard to the threshold for labeling of food and feed products. The potato materials are available from IRMM and its authorized distributors under the European Reference Material brand names ERM-BF421a and ERM-BF421b for the non-GMO and GMO potato materials, respectively.

ABBREVIATIONS USED

AOTF, acousto-optical tunable fluorescence; bp, base pairs; CRM, Certified Reference Material; CTAB, cetyltrimethylammonium bromide; EC, European Commission; ERM, European Reference Material; GM(O), genetically modified (organism); IRMM, Institute for Reference Materials and Measurements; KFT, Karl Fischer titration; kb, kilobase pairs; LOD, limit of detection; M, molecular DNA marker; *N*, number of samples (e.g., CRM vials); *n*, number of subsamples (replicates) analyzed; NIR, near infrared; PCR, Polymerase Chain Reaction; PLS1, partial least squares 1 algorithm; PSA, particle size analysis; *s*, standard deviation; T-DNA, transfer DNA (i.e., the DNA fragment transferred from *Agrobacterium tumefaciens* to the plant genome during genetic transformation); *ugp*, uridine diphosphoglucose pyrophosphorylase gene from *Solanum tuberosum*.

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