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Toward Metrological Traceability for DNA Fragment Ratios in GM Quantification. 2. Systematic Study of Parameters Influencing the Quantitative Determination of MON 810 Corn by Real-Time PCR

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This paper is part of a set of three papers investigating metrological traceability of the quantification of DNA fragments as, for instance, used for quantification of genetic modifications. This paper evaluates the possible impact of several factors on results of real-time Polymerase Chain Reaction (PCR) measurements. It was found that the particle size of the powder samples does not have an influence, whereas the nature of the calibrant (plasmidic or genomic DNA) has a significant effect. Moreover, two real-time PCR detection methods (construct-specific and event-specific) for MON 810 corn were compared. The results obtained in a specifically designed interlaboratory study revealed a significant influence of the DNA extraction method on measurement results when the MON 810 construct-specific real-time PCR detection method was applied. Statistical analyses confirmed the importance of validating DNA extraction methods in conjunction with real-time PCR methods.

KEYWORDS: Genetically modified organism; GMO; DNA; extraction; real-time PCR; PCR efficiency; DNA copy number ratio

INTRODUCTION

In October 2004 the European Commission recommended that the content of genetically modified (GM) food and feed be expressed as the percentage of GM DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of haploid genomes (2004/787/EC) (*1*). Throughout this paper the term "copy number ratio" is used for this quantity. Until publication of this recommendation the GM labeling threshold stipulated in European Regulation (EC) 1829/2003 (*2*) was commonly understood as a mass/mass percentage, that is, mass fraction (*3*). Therefore, currently available Certified Reference Materials (CRMs) of GM food and feed were certified for their mass fraction of ground GM seeds in mixtures with ground non-GM seeds. To support the implementation of Commission Recommendation 2004/787/EC, strategies for the certification of GM CRMs for their copy number ratios using real-time Polymerase Chain Reaction (PCR) need to be developed. Measurement results expressed in copy number ratios require, as any other measured value, metrological traceability to ensure comparability of results in time and between laboratories. Therefore, the prerequisites for reliable quantification of DNA fragments are identification of the factors having an impact on the measurement results and the development of suitable calibrants.

A research strategy was developed by the Institute for Reference Materials and Measurements (IRMM), and the findings were gathered in three papers. In the CCQM-P60 study (part 1 of this series) the impact of DNA extraction methods on real-time PCR was analyzed. In addition, a multifacet interlaboratory comparison, designed and coordinated by IRMM, aimed to investigate the factors influencing the determination of the DNA copy number ratio by real-time PCR (parts 2 and 3 of this series).

A total of 43 laboratories could be selected for this interlaboratory comparison on the basis of proven experience and quality assurance systems in place at each laboratory. Six method combinations were collaboratively trialed to investigate the influence of the DNA extraction method and the real-time PCR detection method on the measured relative copy number of transgenic per endogenous sequences (**Table 1**). Additionally, the impact of the nature of the calibrant was analyzed by using genomic DNA (gDNA) and plasmidic DNA (pDNA) for calibration of the real-time PCR measurements. The influence of the particle size of the unknown sample was addressed through the inclusion of fine and coarse powders. These were prepared by mixing pure GM MON 810 and non-GM corn powders with similar average particle sizes of about 100 and 50 μm for the coarse and fine materials, respectively.

In the current study three different DNA extraction methods, namely, the cetyl trimethyl ammonium bromide (CTAB)

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Table 1. Setup of the Copy Number Certification Study

method combination	no. of data sets	no. of retained data sets	extraction method	detection method[a]	calibrant
1	12	10	CTAB	constr-spec	constr-spec pDNA
1	12	10	CTAB	constr-spec	gDNA
2	11	9	Wizard	constr-spec	constr-spec pDNA
2	11	9	Wizard	constr-spec	gDNA
3	11	10	GENE*Spin*	constr-spec	constr-spec pDNA
3	11	10	GENE*Spin*	constr-spec	gDNA
4	11	10	CTAB	event-spec	event-spec pDNA
4	11	10	CTAB	event-spec	gDNA
5	11	10	Wizard	event-spec	event-spec pDNA
5	11	10	Wizard	event-spec	gDNA
6	11	10	GENE*Spin*	event-spec	event-spec pDNA
6	11	10	GENE*Spin*	event-spec	gDNA

[a] Constr-spec and event-spec refer to the construct-specific and event-specific real-time PCR detection methods.

method (*4*), the Wizard genomic DNA purification kit (Promega Benelux, Leiden, The Netherlands), and the GENE*Spin* kit (GeneScan Analytics GmbH, Freiburg, Germany), were compared with respect to their possible impact on the determination of the copy number ratio. Two real-time PCR detection methods, either construct- or event-specific, were evaluated. Whereas initially mostly construct-specific detection methods were developed (*5*), the increasing number of GM events authorized in Europe led to the decision to favor event-specific real-time PCR detection methods (*6*) because of their ability to unambiguously identify the specific GM event. Both PCR methods used in this study passed successfully collaborative method validations according to ISO 21570 (*7*, *8*). It has to be noted that matrix CRMs were used in these validations as unknown samples as well as for calibration and that only one DNA extraction method was applied during the validation of each method. However, ISO 21570 (*7*) claims that also other DNA extraction methods can be used provided they can produce the same results.

In the current study, gDNA and pDNA calibrants were used to calibrate the two different real-time PCR methods quantifying the DNA copy number ratio. The use of pDNA calibrants for real-time PCR assays is relatively new (*9*). More recently, pDNA calibrants containing two (*10*) or more (*11*) target sequences have been developed. The pDNA calibrants applied in this study were multiple-target plasmids (developed by Nippon Gene, Toyama, Japan) or dual-target plasmids (developed by IRMM).

MATERIALS AND METHODS

Experimental Details. Two coarse and two fine powder materials were produced at IRMM containing 1.5, 4.5, 0.8, and 3.8 m/m % GM, respectively. These GM powders were prepared gravimetrically by dry-mixing of MON 810 GM powder and non-GM powder with verified purity and similar particle size distribution. Consequently, the GM values given above refer to mass fractions expressed in percent. Three DNA extraction methods were evaluated, namely, the CTAB method, the Wizard kit, and the GENE*Spin* kit. Two real-time PCR detection methods were applied: a construct-specific and an event-specific method. Using the construct-specific detection method, fragments of the *zSSIIb* gene (*12*) and the *hsp70/cryIA(b)* junction specific for the endogenous and transgenic targets, respectively, were amplified (*11*). The event-specific detection method targeted fragments of the endogenous *hmg* gene (*13*) and the junction between the integration-border region of the plant genomic sequence and the inserted sequence element originating from the cauliflower mosaic virus 35S promoter (plant/P35S junction) (*14*), the latter specific for hybrid corn event MON 810 (*7*). pDNA and gDNA were used as calibrants for all real-time PCR measurements. The gDNA calibrant was extracted from young leaves of verified MON 810 GM plants. Two plasmids, one carrying besides the targeted construct-specific sequence the taxon-specific sequence (Nippon Gene, Toyama, Japan) and the other one carrying besides the targeted event-specific sequence the taxon-specific sequence (developed by IRMM), were used specifically for the two selected detection methods as calibrants.

The study aimed to have each method combination of extraction and detection measured by at least 10 different laboratories. To test the six possible method combinations, a minimum of 60 data sets was envisaged for the study. A total of 43 laboratories specialized in the field of GM detection were worldwide selected, and a total of 268 real-time PCR experiments were carried out and evaluated. Finally, each method combination was covered by 11 individual data sets with the exception of method combination 1, for which 12 individual data sets could be collected (**Table 1**).

Overview of Experiments Performed for the Analysis of One Data Set. To obtain from each laboratory data under reproducibility conditions, the experiments were spread over at least 2 days (further referred to as days 1 and 2). On day 1 DNA extraction was carried out on four unknown powder samples (U1−U4) using one of the three DNA extraction methods. The extracted DNA was quantified by measuring the absorption at 260 nm and (if possible) by the PicoGreen dsDNA Assay Kit. Real-time PCR measurements were carried out on two 96 well plates. On day 2 the analyses of day 1 were independently repeated using another set of samples (U5−U8) from the same unknown GM powders.

Reagents, Kits, and Consumables. Participating laboratories had to prepare buffers for the CTAB DNA extraction method. Laboratories were provided with the Wizard genomic DNA purification kit (Promega, Benelux, Leiden, The Netherlands) and the GENE*Spin* kit (GeneScan Analytics GmbH, Freiburg, Germany). TaqMan primers, probes, and Universal PCR Master Mix (Applied Biosystems, Foster City, CA), nuclease-free water (Promega Benelux), the PicoGreen dsDNA Assay Kit (Molecular Probes Europe, Leiden, The Netherlands), the GM Maize Detection Plasmid Set (Nippon Gene, Toyama, Japan), and the event-specific dual-target plasmid were provided by IRMM. All reagents, kits, DNA solutions, and consumables were shipped to the participating laboratories on dry ice.

Preparation of MON 810 Powders Used as Unknowns. MON 810 certified seeds from the first-generation cultivar DK 513 were delivered by R.a.g.t. Semences, Rodez, France. From them were produced coarse powder materials according to IRMM's protocol for the preparation of dry-mixed corn CRMs. The dried starting materials were ground using a high-impact mill for two grinding steps. Particle size measurements were performed with a particle size analyzer (PSA, Sympatec, Clausthal-Zellerfeld, Germany). The median particle size and span of the coarse powder materials was 112 ± 3 μm for the non-GM powder and 105 ± 3 μm for the GM powder. A 10 m/m % powder was prepared by mixing of the MON 810 GM powder with the non-GM powder. The 1.5 and 4.5 m/m % materials were obtained by serial dilutions with non-GM powder. Further details concerning the processing of corn powders with different GM mass fractions can be found, for example, in Trapmann et al. (*15*).

Fine powder materials were prepared as described by Trapmann et al. (*16*). Dried starting materials were ground using a high-impact mill. Grinding to smaller particle sizes was completed with a second grinding step using liquid nitrogen cooling. The median particle size and span of the fine powder materials were 47 ± 3 μm for the non-GM powder and 60 ± 3 μm for the GM powder. A 10 m/m % powder was prepared by mixing of the MON 810 GM powder with the non-GM powder. The 0.8 and 3.8 m/m % materials were obtained by serial dilution with the non-GM powder.

Preparation of gDNA Calibrant. Prior to the large-scale extraction of genomic DNA from plants, the MON 810 positive status of each individual plant was confirmed by PCR. For this reason, genomic DNA was extracted from leaves collected separately from each plant by using a rapid DNA extraction method (*17*). DNA was dissolved in 100 μL of 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). A 170 bp

Table 2. Oligonucleotides Used in This Study

name	orientation	sequence	amplicon size (bp)	ref
		Qualitative PCR for Cloning of Event-Specific Fragments		
plant/P35S junc				
VW01	forward	5′-TCG AAG GAC GAA GGA CTC TAA CG-3′	170	7
VW03	reverse	5′-TCC ATC TTT GGG ACC ACT GTC G-3′		
hmg				
ZmIIF	forward	5′-GAT TCC CCT CTC CTG GTC GA-3′	351	*a*
ZmIIR	reverse	5′-CAA CAC ATG GTT CAG TAA GCA TAC G-3′		
		Quantitative Construct-Specific Method		
hsp70/cryIA(b)				
M810 2-5′	forward	5′-GAT GCC TTC TCC CTA GTG TTG A-3′	113	*11*
M810 2-3′	reverse	5′-GGA TGC ACT CGT TGA TGT TTG-3′		
M810-Taq	probe	5′-(FAM)-AGA TAC CAA GCG GCC ATG GAC AAC AA-(TAMRA)-3′		
zSSIIb				
SSIIb 1-5′	forward	5′-CTC CCA ATC CTT TGA CAT CTG C-3′	151	*11*
SSIIb 1-3′	reverse	5′-TCG ATT TCT CTC TTG GTG ACA GG-3′		
SSIIb-Taq	probe	5′-(FAM)-AGC AAA GTC AGA GCG CTG CAA TGC A-(TAMRA)-3′		
		Quantitative Event-Specific Method		
plant/P35S junc				
Mail-F1	forward	5′-TCG AAG GAC GAA GGA CTC TAA CGT-3′	92	*7*
Mail-R1	reverse	5′-GCC ACC TTC CTT TTC CAC TAT CTT-3′		
Mail-S2	probe	5′-(FAM)-AAC ATC CTT TGC CAT TGC CCA GC-(TAMRA)-3′		
hmg				
ZM1-F	forward	5′-TTG GAC TAG AAA TCT CGT GCT GA-3′	79	*7*
ZM1-R	reverse	5′-GCT ACA TAG GGA GCC TTG TCC T-3′		
ZM1	probe	5′-(FAM)-CAA TCC ACA CAA ACG CAC GCG TA-(TAMRA)-3′		

[a] Primers were designed in-house on the basis of the sequence reported by Krech et al. (*13*).

fragment of the plant/P35S junction specific for corn event MON 810 was amplified using PCR primers VW01/sense and VW03/antisense (**Table 2**).

The final large-scale gDNA extraction from 1 g of verified GM leaves of corn MON 810 was performed using the QIAGEN DNeasy Plant Maxi Kit (QIAGEN, Hilden, Germany). gDNA was eluted with 500 μL of AE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0), and 82 extracts were pooled in total. A volume of 400 μL of gDNA was provided to the participating laboratories to be used as the gDNA calibrant in the real-time PCR experiments.

Construction of the Event-Specific Dual-Target Plasmid. A 170 bp fragment of the plant/P35S junction was amplified using corn gDNA, isolated from pure GM powder, and the VW01/sense and VW03/antisense primers (**Table 2**). The resulting fragment was cloned in pT-Adv, making use of the TA overhangs generated by Taq polymerase (Invitrogen, Carlsbad, CA), and subsequently subcloned in pUC18. Additionally, a 351 bp fragment of the endogenous high mobility group (*hmg*) gene from corn was amplified using the ZmIIF/sense and ZmIIR/antisense primers (**Table 2**). The amplicon was cloned in pCR2.1 (Invitrogen, Carlsbad, CA), and the plasmid was digested with *Hin*dIII and *Xba*I to release the fragment specific for the *hmg* gene. Subsequently, this fragment was ligated in the *Hin*dIII/*Xba*I restricted pUC18 derived plasmid containing the plant/P35S junction using the Rapid DNA ligation kit (Roche, Mannheim, Germany) to yield the dual-target plasmid.

Sequencing of the dual-target plasmid was carried out using a Beckman Coulter CEQ8000 system, according to the protocol of the Dye Terminator Cycle Sequencing (DTCS) kit (Beckman Coulter, Fullerton, CA). It confirmed the presence and correctness of the two sequences targeted by the event-specific PCR method in the 3309 bp plasmid. Standard cloning techniques were used as described by Sambrook et al. (*18*).

Preparation of pDNA Calibrants. The dual-target plasmid, specific for the event-specific detection method, was isolated from a 5 L overnight culture in Luria broth medium containing ampicillin (100 μg/mL) using the QIAGEN Plasmid Giga Kit (QIAGEN). A volume of 160 μL of pDNA solution (nominal 2×10^6 cp/μL) was provided to the participants. A construct-specific GM Maize Detection Plasmid Set was purchased from Nippon Gene (Toyama, Japan). Both pDNA calibrants were provided and used in the nonlinearized form.

DNA Extraction from Unknown Powder Samples. For method combinations 1 and 4 (**Table 1**), gDNA was extracted from 100 mg of the unknown powder samples according to a CTAB-based method (*4*). DNA pellets were finally dissolved in 100 μL of nuclease-free water. For method combinations 2 and 5, genomic DNA was isolated from 5×20 mg of the unknown powder samples using the Wizard genomic DNA purification kit. DNA pellets originating from one unknown powder sample were each dissolved in 30 μL of nuclease-free water and pooled. gDNA was extracted from 200 mg of unknown powder samples using the GENE*Spin* kit for method combinations 3 and 6. gDNA was eluted in 2×100 μL of elution buffer CE (Tris-based buffer).

DNA Quantification. The DNA concentration was estimated by measuring the absorption at 260 nm (A_{260}) and by using the PicoGreen dsDNA Assay Kit. The measurement unit, ng/μL, of the DNA concentration was converted into cp/μL by applying the following formulas:

$$1 \text{ ng of DNA} = 0.97 \times 10^{12} \text{ bp} \quad (1)$$

gDNA calibrant, endogenous target sequence:

$$\text{copy number (cp/}\mu\text{L)} = \frac{\text{DNA concentration (ng/}\mu\text{L)} \times 0.97 \times 10^{12} \text{ (bp/ng)}}{\text{haploid genome size (bp/cp)}} \quad (2)$$

gDNA calibrant, transgenic target sequence:

copy number of transgenic sequences calculated using eq 2 must be divided by 2 because of the heterozygous nature of MON 810 hybrid corn

pDNA calibrant:

$$\text{copy number (cp/}\mu\text{L)} = \frac{\text{DNA concentration (ng/}\mu\text{L)} \times 0.97 \times 10^{12} \text{ (bp/ng)}}{\text{plasmid size (bp/cp)}} \quad (3)$$

with plasmid size = 3309 bp.

As the genome size of corn is known to vary by up to 36% (*19*), a size of 2425 Mbp for the haploid corn genome (*20*) was assumed for the conversion of DNA concentrations (ng/μL) into copy numbers per microliter, which is within the boundaries indicated (*19*).

Real-Time PCR Methods. For the detection of the endogenous target of the construct-specific method, a 151 bp fragment of the *Zea* starch synthase IIb (z*SSIIb*) gene was amplified using the forward primer SSIIb 1-5′, reverse primer SSIIb 1-3′, and probe SSIIb-Taq. A 113 bp fragment covering the junction region between the heat shock protein 70 gene and the *Bacillus thuringiensis* gene *cryIA(b*) [*hsp70*/*cryIA(b*)] of MON 810 was amplified using the sense primer M810 2-5′, anti-sense primer M810 2-3′, and probe M810-Taq (**Table 2**).

The presence of the endogenous target of the event-specific method was assessed through amplification of a 79 bp fragment of the *hmg* gene using the ZM1-F sense primer, ZM1-R antisense primer, and ZM1 probe. A 92 bp fragment of the plant/P35S junction was amplified using the sense primer Mail-F1, anti-sense primer Mail-R1, and probe Mail-S2 (**Table 2**).

Dilution series were prepared from gDNA in nuclease-free water ranging from 2×10^4 to 2 cp/μL and for the event-specific pDNA ranging from 1×10^5 to 2 cp/μL in a background of ColE1 pDNA (1 ng/μL). The dilution series for the construct-specific plasmid, containing five different concentrations, was provided by Nippon Gene (Toyama, Japan). Additionally, two further dilutions (2×10^4 and 2 cp/μL, respectively) were prepared by the participating laboratories in a background of ColE1 pDNA (.5 ng/μL).

TaqMan Universal PCR experiments were carried out according to the manufacturer's instructions (Applied Biosystems) with all runs performed for 45 cycles.

Criteria for the Exclusion of Data Sets. Different parameters were assessed to establish data exclusion criteria for the current study. These included the PCR efficiency estimated on the basis of the slope of the calibration curve, the correlation coefficient of the calibration curve, PCR inhibition analyses, and confirmed technical mistakes. Control limits were defined on the basis of the slopes of pDNA and gDNA calibrants. The average of all calibration slopes was -3.53, and the standard deviation was 0.20. The PCR efficiency estimated for the average slope was $94 \pm 6\%$ (1 SD). The lower and higher control limits defined as the average PCR efficiency of the study $\pm 3 \times$ standard deviation (3 SD) were 74 and 113%, respectively.

As two independent analyses were carried out, one data set comprised a total of eight calibration curves consisting of the detection of the endogenous and transgenic targets using either a gDNA or a pDNA calibrant. When one calibration slope of a total of eight slopes gave a value outside the control limits, the entire data set was excluded for subsequent analyses. On the basis of this exclusion criterion, four data sets were excluded for the construct-specific and one was excluded for the event-specific detection method.

A value for the correlation coefficient below 0.98 was considered, in accordance with the method validation guidelines of the Community Reference Laboratory for GM Food and Feed (*21*), as a second criterion for exclusion of an entire data set. One data set of the construct-specific detection method was excluded because of a correlation coefficient of 0.96 obtained for the gDNA calibrant.

One data set of the event-specific method was excluded due to the occurrence of technical problems with the real-time PCR instrument of the participating laboratory. Another data set was not retained because of an obvious mixup of the unknown powder samples on the second day of the analysis for the event-specific method.

In total, five data sets were excluded for the construct-specific and three for the event-specific detection method.

A third exclusion criterion concerned anomalies related to the dilution of unknown powder samples. Unknown powder samples were assayed undiluted, 2× diluted, and 5× diluted. Diluted DNA samples that exhibited Ct values beyond the linear working range of the calibration curve for the detection of the transgenic target were excluded. Consequently, any bias introduced by the DNA quantification method has no influence on the copy number ratio. Moreover, diluted unknown powder samples were excluded when a mistake in the dilution had been reported. The measurement result corresponding to the undiluted sample was in that case taken as the average copy number ratio.

Table 3. Overview of Average Copy Number Ratios of Undiluted, 2× Diluted, and 5× Diluted Samples for 4.5% Mass Fraction Powder for Each DNA Extraction and Real-Time PCR Detection Method, Obtained Using either a gDNA or pDNA Calibrant

detection method	calibrant	DNA extraction method	copy no. ratio undiluted	2× diluted	5× diluted
constr-spec	gDNA	CTAB	3.4	3.4	3.7
		Wizard	2.9	2.9	3.1
		GENE*Spin*	2.6	2.6	2.7
		all combined	3.0	3.0	3.2
constr-spec	pDNA	CTAB	2.9	2.9	3.1
		Wizard	2.4	2.4	2.5
		GENE*Spin*	2.3	2.3	2.4
		all combined	2.6	2.5	2.7
event-spec	gDNA	CTAB	2.8	2.9	3.0
		Wizard	2.7	2.9	2.9
		GENE*Spin*	2.8	2.9	2.9
		all combined	2.8	2.9	2.9
event-spec	pDNA	CTAB	2.2	2.3	2.2
		Wizard	2.3	2.4	2.4
		GENE*Spin*	2.4	2.5	2.4
		all combined	2.4	2.4	2.4

The possible occurrence of PCR inhibition effects was assessed. Copy number ratios were calculated for each combination of DNA extraction and real-time PCR method for the undiluted, 2× diluted, and 5× diluted samples of the 4.5 m/m % powder. Average values obtained using different real-time PCR detection methods (construct-specific or event-specific detection method) and calibrants (pDNA or gDNA calibrant) are compared in **Table 3**. The differences between the average copy number ratios measured on the various dilutions remained within the limits of variation for real-time PCR detection methods (relative standard deviation between 15 and 25%). Therefore, no individual measurement result was excluded on this basis.

Statistical Analyses. Statistical analysis was performed using Statistica 7.0 software (Statsoft, Tulsa, OK). The distribution of the copy number ratios was checked, whereby the results for the analyses spread over 2 days were combined. Normal probability plots revealed a logarithmic distribution for each mass fraction, and all data were therefore log-normalized using the following formula:

$$\text{log-normalized copy number ratio} = \log(\text{copy number ratio/mass fraction}) \text{ with the mass fraction} = 0.8, 1.5, 3.8, \text{ and } 4.5\%, \text{ respectively} \quad (4)$$

The measurement results reported in part 1 of this publication series led to a smaller data set than reported here. For that study, no deviation from the unimodality and normal distribution of the data could be confirmed, and therefore no log-normal transformation was needed.

Probability plots for the log-normalized values using pDNA and gDNA calibrants and comprising all unknowns showed near-normal distributions. Moreover, log-normalized values for the copy number ratio were plotted per method combination to investigate the occurrence of trends.

Main effects and factorial ANOVA were used to investigate the main factors that may have an influence on the determination of a measurement result. In a factorial ANOVA both the main factors and the interactions between factors were studied. ANOVA was conducted using log-normalized copy number ratios originating from all method combinations.

Subsequently, the data were grouped per calibrant and method combination, and main effects and factorial ANOVA were performed for each calibrant. For both analyses, the copy number ratio was selected as a dependent variable and the extraction method, detection method, and particle size were selected as categorical factors.

RESULTS

The copy number ratio was calculated as the average determined for undiluted, 2× diluted, and 5× diluted samples,

Table 4. Average Copy Number Ratio for Each Method Combination and Mass Fraction

method combination	mass fraction (%)	DNA calibrant	av copy no. ratio	standard deviation	relative standard deviation (%)
1	0.8	gDNA	0.7	0.2	28
2	0.8	gDNA	0.6	0.2	28
3	0.8	gDNA	0.5	0.1	27
4	0.8	gDNA	0.5	0.1	27
5	0.8	gDNA	0.6	0.2	33
6	0.8	gDNA	0.5	0.1	26
1	0.8	pDNA	0.5	0.1	21
2	0.8	pDNA	0.5	0.1	16
3	0.8	pDNA	0.4	0.1	22
4	0.8	pDNA	0.4	0.1	22
5	0.8	pDNA	0.4	0.1	26
6	0.8	pDNA	0.5	0.1	18
1	1.5	gDNA	1.2	0.2	22
2	1.5	gDNA	1.1	0.2	17
3	1.5	gDNA	0.9	0.2	19
4	1.5	gDNA	1.0	0.3	28
5	1.5	gDNA	1.0	0.4	39
6	1.5	gDNA	1.0	0.3	27
1	1.5	pDNA	0.9	0.2	21
2	1.5	pDNA	0.8	0.1	17
3	1.5	pDNA	0.8	0.1	14
4	1.5	pDNA	0.8	0.1	16
5	1.5	pDNA	0.8	0.2	22
6	1.5	pDNA	0.8	0.1	16
1	3.8	gDNA	2.9	0.5	18
2	3.8	gDNA	2.5	0.4	15
3	3.8	gDNA	2.2	0.3	15
4	3.8	gDNA	2.4	0.5	20
5	3.8	gDNA	2.4	0.6	24
6	3.8	gDNA	2.3	0.4	19
1	3.8	pDNA	2.3	0.4	15
2	3.8	pDNA	2.0	0.3	16
3	3.8	pDNA	2.0	0.2	9
4	3.8	pDNA	2.0	0.3	15
5	3.8	pDNA	2.0	0.3	16
6	3.8	pDNA	2.1	0.3	13
1	4.5	gDNA	3.4	0.5	13
2	4.5	gDNA	3.0	0.4	15
3	4.5	gDNA	2.6	0.4	15
4	4.5	gDNA	2.9	0.6	20
5	4.5	gDNA	2.8	0.8	29
6	4.5	gDNA	2.8	0.9	30
1	4.5	pDNA	2.9	0.6	20
2	4.5	pDNA	2.4	0.4	16
3	4.5	pDNA	2.4	0.4	15
4	4.5	pDNA	2.5	0.4	16
5	4.5	pDNA	2.4	0.4	17
6	4.5	pDNA	2.4	0.4	16

provided the results were within the calibration range. For each method combination the average copy number ratio was calculated for the mass fraction of the unknown samples of 0.8, 1.5, 3.8, and 4.5%, respectively (**Table 4**). For each unknown sample the average copy number ratios obtained with gDNA and pDNA calibrants were plotted (**Figure 1**). It was noted that the results obtained using a pDNA calibrant showed a smaller standard deviation than those originating from gDNA calibration curves. This indicates that calibration with gDNA was less robust within the given experimental setup. **Figure 1** revealed a high degree of variation for the copy number ratios resulting from the construct-specific detection method. There seems to be a dependence of the obtained value on the DNA extraction method for this approach. On the contrary, the event-specific detection method did not show this effect.

A recurring effect was noted from plotting the log-normalized copy number ratio for each mass fraction (**Figure 2**). Log-normalized values obtained using pDNA calibrants were lower compared to those obtained with gDNA calibrants.

Main effects ANOVA revealed a significant impact of the extraction method (probability $p = 6 \times 10^{-10}$), the detection method ($p = 5 \times 10^{-4}$), and the calibrant ($p = 0$) on the copy number ratio, whereas there was no significant effect noted for the difference in particle size (fine versus coarse powders, $p = 0.4$). Furthermore, two independent factorial ANOVA analyses were performed using as a dependent variable the copy number ratio but differing in the categorical factors used. For the first analysis the extraction method, calibrant, and particle size were selected as categorical factors, whereas the extraction method, detection method, and particle size were the categorical factors for the second factorial ANOVA. In both cases there was a significant influence of the extraction method ($p = 9 \times 10^{-8}$ and $p = 7 \times 10^{-9}$, respectively), but no impact from the particle size ($p = 0.5$ for both analyses). The calibrant ($p = 0$) and the detection method ($p = 2 \times 10^{-4}$) also contributed to the variation of the copy number ratio. Subsequently, data were grouped either per pDNA or per gDNA calibrant. The copy number ratio was selected as a dependent variable, and the extraction method, detection method, and particle size were selected as categorical factors. A significant influence of the extraction method ($p = 1 \times 10^{-4}$ and $p = 4 \times 10^{-7}$, respectively) and the detection method ($p = 2 \times 10^{-2}$ and $p = 2 \times 10^{-3}$, respectively) was found. The effect of the particle size ($p = 0.7$ and $p = 0.5$, respectively) on the variation of the copy number ratio was negligible.

In addition, data were grouped per detection method to assess the impact of the calibrant, the extraction method, and the particle size on the copy number ratio. For the event-specific detection method, main effects ANOVA revealed a significant influence of the calibrant ($p = 2 \times 10^{-15}$), whereas there was no notable contribution from the particle size ($p = 0.2$) and the extraction method ($p = 0.7$). Factorial ANOVA confirmed the findings from main effects ANOVA concerning the main factors and did not show a significant interaction between the extraction method and the calibrant ($p = 0.3$), between the extraction method and the particle size ($p = 0.7$), between the calibrant and the particle size ($p = 0.96$), or between the extraction method, the calibrant, and the particle size ($p = 0.96$), respectively. As already suspected from the differences in average copy number ratios for method combinations 1−3 of the construct-specific detection method (**Figure 1**), main effects ANOVA revealed a significant influence of the extraction method ($p = 0$) as well as of the calibrant ($p = 0$). The contribution from the particle size ($p = 0.9$) was negligible. Factorial ANOVA confirmed the observations from main effects ANOVA concerning the factors influencing the copy number ratio. Moreover, an assessment of the interactions between factors showed that there was a significant interaction between the calibrant and the extraction method ($p = 5 \times 10^{-2}$). There was no significant influence from the interaction between the extraction method and the particle size ($p = 0.8$), between the calibrant and the particle size ($p = 0.7$), or between the extraction method, the calibrant, and the particle size ($p = 0.9$), respectively.

Copy number ratios, obtained using either gDNA extracted from verified MON 810 plants or the dual-target plasmid as a calibrant for the event-specific quantitative PCR, were independent from the extraction method and the particle size (**Figure 3A**). Moreover, there was no significant influence from the interaction between the extraction method and the particle size

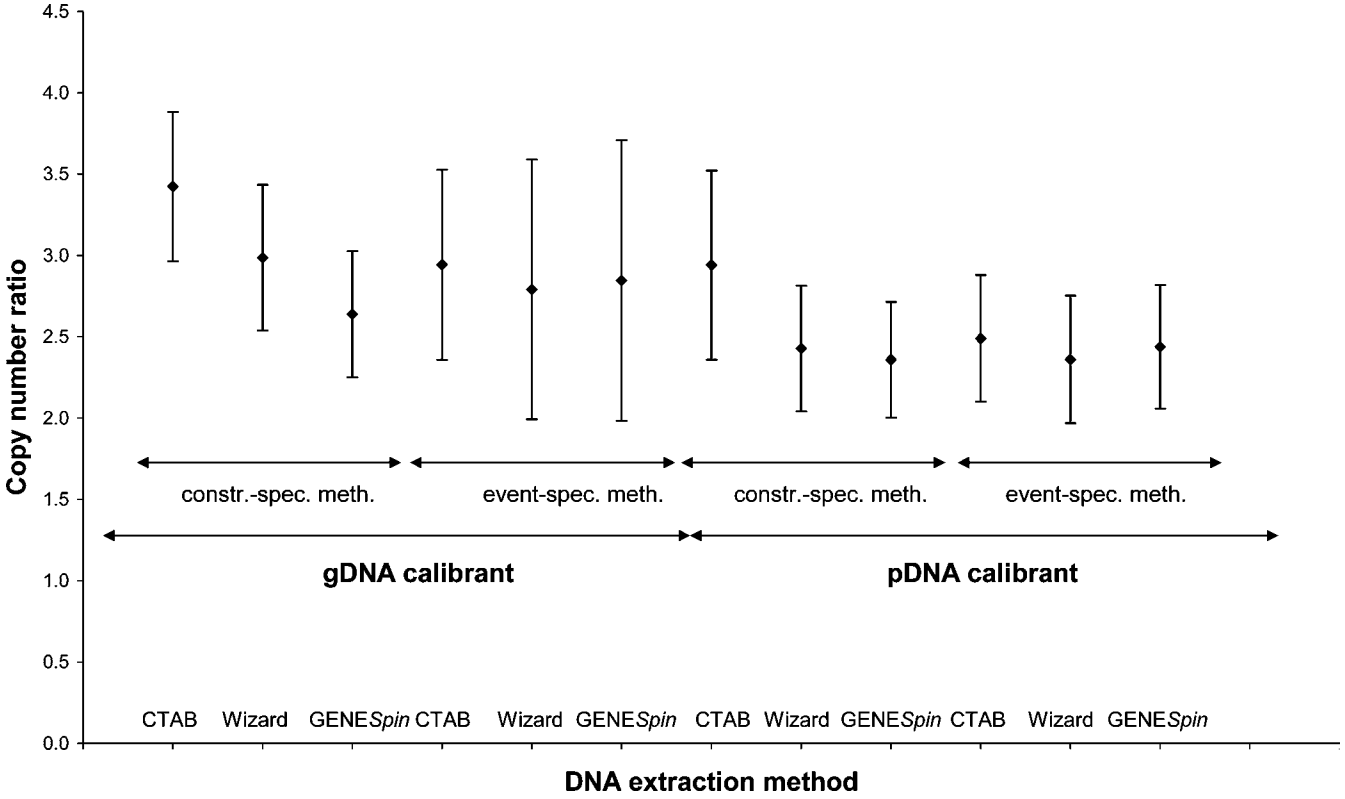

Figure 1. Comparison of copy number ratios measured for 4.5% mass fraction material using different DNA extraction and real-time PCR method combinations: CTAB, Wizard, and GENE*Spin* refer to the DNA extraction methods, constr.-spec. meth. and event-spec. meth. refer to the construct-specific and event-specific real-time PCR detection methods. Average results and their standard deviation for the copy number ratios represent 10 data sets for method combinations 1 and 3–6 and 9 data sets for method combination 2 (**Table 1**).

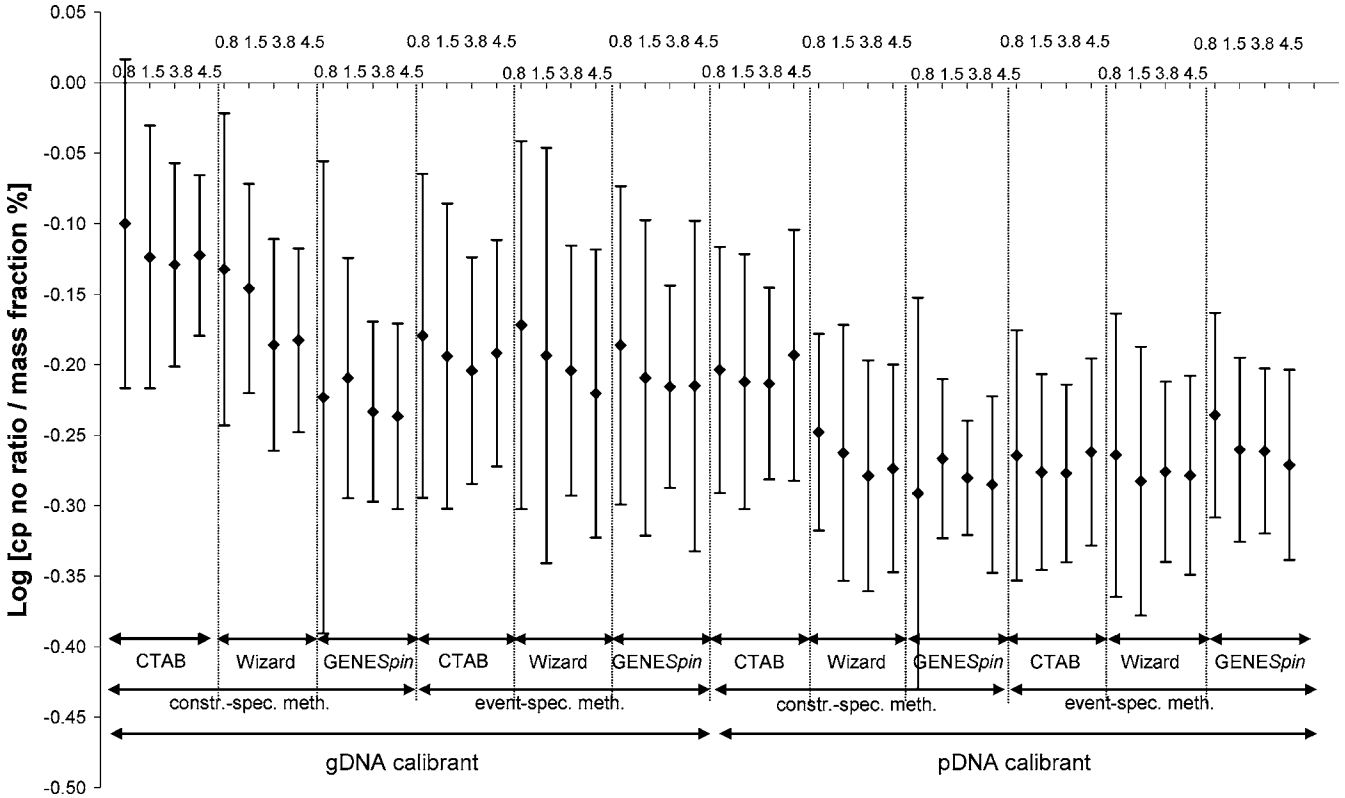

Figure 2. Evaluation of log-normalized copy number (cp no) ratios grouped per method combination (**Table 1**). The corresponding average values were plotted relative to the mass fraction of the unknown samples for each calibrant with vertical bars representing the standard deviation.

(**Figure 3A**). Statistical analyses performed for each calibrant using the construct-specific detection method revealed a significant impact from the extraction method on the copy number ratio (**Figure 3B**). There was no contribution to this variation

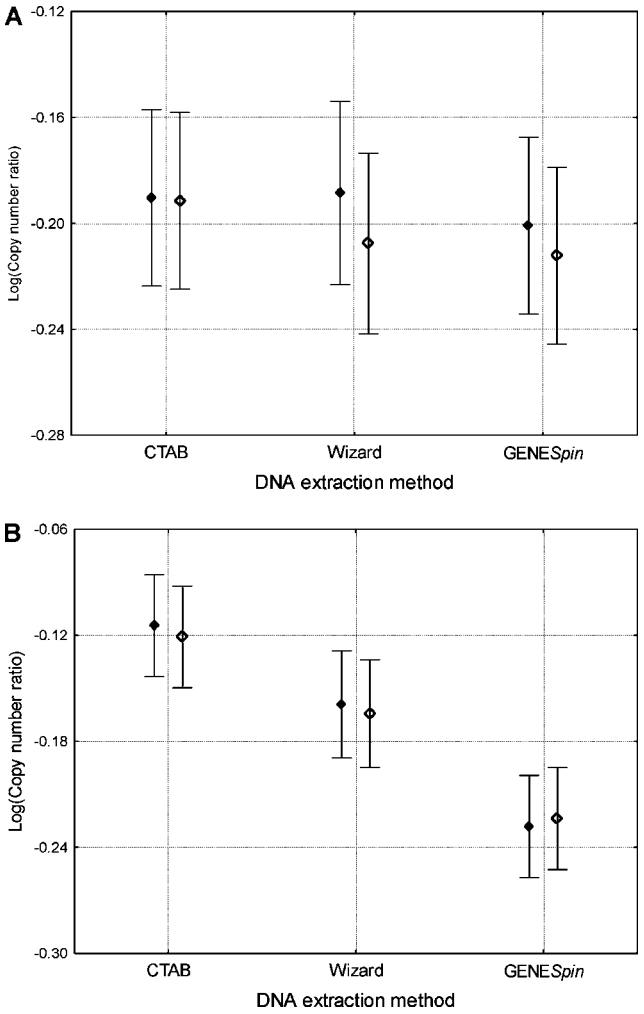

Figure 3. Factorial ANOVA to investigate the interactions between factors influencing the determination of the copy number ratio for the (**A**) event- and (**B**) construct-specific detection method using a gDNA calibrant. Average copy number ratios are depicted relative to the mass fraction of the unknown fine (◆) and coarse (◇) powder samples for each method combination. Vertical bars denote the 95% confidence interval.

from the particle size or from the interaction between the extraction method and the particle size (**Figure 3B**).

Both statistical analyses (main effects and factorial ANOVA) and graphical evaluation of log-normalized data (**Figure 1**) showed that in the case of the construct-specific real-time PCR detection method the resulting copy number ratio was influenced by the DNA extraction method applied for the unknown powder samples. Within the given experimental setup the construct-specific real-time PCR detection method was less robust than the event-specific detection method, with which no effect from the DNA extraction method was observed. It has to be noted that the study did not provide sufficient evidence that the latter statement could be generalized.

DISCUSSION

The results of the interlaboratory comparison presented here allow a number of conclusions about the impact of several factors on the quantification of GM by real-time PCR. Previous studies have addressed some of these issues, but never altogether in a systematic manner considering also variations from between-laboratory performance. Taking into account the general variability of PCR measurement results, a large data pool had to be created to test differences for statistical significance.

Regarding the impact of various extraction methods, Smith and Maxwell (*22*) determined the relative concentration of an endogenous corn invertase (*ivr1*) sequence by real-time PCR as a means to compare four different DNA extraction methods with respect to the overall quality and quantity of DNA isolated from lightly processed and severely degraded food products. They suggested that the extraction efficiency was the most important factor influencing amplification of the *ivr1* gene by real-time PCR. Peano et al. (*23*) have shown that the DNA extraction method had an influence on the "quality" (integrity and purity) and quantity of extracted DNA. Moreover, they investigated the influence of the DNA extraction method on the quantification of corn MON 810 and Roundup Ready soybean CRMs by real-time PCR through comparison of the measured values with those expected. They proposed to use the DNA extraction method that gives the best correlation with the performance of real-time PCR. A recent study called CCQM-P60, organized by IRMM, assessed the impact of the DNA extraction method, the DNA quantity and quality, PCR inhibition, and real-time PCR detection method on the determination of the GM mass fraction of Bt176 (part 1 of this series). It was shown within this study that the quality of extracted gDNA was dependent on both the specific procedure performance of each laboratory and the DNA extraction method applied. Moreover, the occurrence of PCR inhibition for less diluted samples was noted, which resulted in an underestimation of the true value for the investigated GM model.

Altogether these studies suggested that the DNA extraction method has an influence on the quality and quantity of isolated gDNA amenable to real-time PCR amplification.

The interlaboratory comparison presented here employed systematically DNA extraction methods that differ with respect to the cellular lysis, precipitation, and removal of proteins and polysaccharides. The study reveals a dependence of the real-time PCR measurement results on the DNA extraction method applied in the case of the construct-specific real-time PCR detection method. Consequently, the robustness of each real-time PCR method toward DNA extraction has always to be carefully investigated. The current practice to validate a complete measurement procedure for DNA in food or feed samples by incorporating a single DNA extraction method into the validation does only allow the assessment of this specific combination of extraction and detection method without further generalization.

A second factor that was addressed here concerns the particle size of the powder samples to be analyzed. The experiments were performed on fine and coarse powders, whereby it has to be stressed that for each mixture the average particle size of GM and non-GM starting materials was similar to exclude the over- or underestimation of the GM percentage caused by starting materials exhibiting a different particle size (*24*). Our results indicate that the DNA copy number ratio measured by real-time PCR is not influenced by different particle sizes as long as it is assured that GM and non-GM material have a similar particle size.

Another important aspect for quantification is calibration. In a comparative study of genomic, single-target, and multiple-target plasmidic DNA calibrants, the authors considered all three calibrants to be suitable for relative quantification of Roundup Ready soybean (*25*). Their approach differed from the current study with respect to the quantification method (delta Ct method versus standard curve method) and the selected matrix material

(homozygous Roundup Ready soybean versus heterozygous MON 810 corn). The reported relative standard deviations (RSD) were quite high, ranging from 13 to 61%, from 11 to 38%, and from 13 to 36% for genomic, single-target, and multiple-target pDNA calibrants, respectively, measuring GM mass fractions between 0.1 and 5 m/m %. In the current study, there was a significant difference between the measurement results obtained using either a gDNA or a pDNA calibrant. The measurement results originating from plasmidic calibration were lower compared to those obtained with gDNA calibration curves. The reason for that has been investigated. One of the requirements for obtaining true values is that the calibrant should have a similar analytical behavior as the real sample under investigation. The suitability aspect of calibrants is addressed in part 3 of this set of papers.

The choice of the PCR detection method could also have an impact on the measurement results. Both detection methods applied in this study, a construct-specific and an event-specific, were validated before in collaborative trials (*7*, *8*), but only one DNA extraction method was applied for each detection target during the collaborative trial validation. The construct-specific detection method was validated using the QIAGEN DNeasy Plant Maxi kit for DNA extraction. The event-specific detection method was validated using the GENE*Spin* kit. The measurements reported in these validation studies were carried out on corn material with different MON 810 corn mass fractions. For both PCR quantification methods, the absence of other GM events was confirmed by the lack of cross-reactivity with Bt176, Bt11, GA21, T25, and GTS 40-3-2 soybean. Therefore, they should lead to the same measurement results, but the data obtained in this study revealed a significant difference between the real-time PCR detection methods. Like the name says, the construct-specific method targets a sequence specific for the junction region within the construct. In principle, higher values for the construct-specific method could be caused by a contamination of MON 810 corn with another GM event carrying the same construct, for instance, MON 802. There is, however, no evidence for such a contamination (*16*). Therefore, it was concluded that the observed effect was due to the dependence of the measurement result of the construct-specific method on the DNA extraction method.

A new aspect of the study presented here consists of the assessment of the interactions between different factors influencing GM quantification, because this has never been performed until now. Main effects ANOVA and factorial ANOVA demonstrated a significant impact of the extraction method, the detection method, and the calibrant on the DNA copy number ratio. Moreover, a significant interaction between the DNA extraction method and the detection method could be identified. Grouping the data per real-time PCR detection method revealed a significant interaction between the DNA extraction method and the calibrant for the construct-specific real-time PCR detection method. Consequently, the observations in this study indicate that the splitting of the whole GM quantification procedures into nonrelated extraction and PCR steps as used, for example, in ISO 21571 (*26*) and ISO 21570 (*7*), implying a modularity of the complete procedure, is not necessarily correct in all cases. The results obtained both in the CCQM-P60 study, which targeted Bt176 corn (part 1 of this series), and in this study measuring corn MON 810 do not justify the proposed modular approach (*27*). Obviously, the hypothesis that an influence of each step of the analytical procedure on the following steps can be avoided was not supported for the procedures used by both interlaboratory studies. Therefore, adequate method validation ideally requires the investigation of the combination of DNA extraction and real-time PCR. Consequently, this is also a prerequisite to establish metrological traceability of the measurement results on food and feed samples.

The current study aimed to assess the factors influencing the measurement procedure for GM quantification in an interlaboratory setup. To establish general performance criteria, it was important to obtain information on the robustness of the DNA extraction and real-time PCR methods and the method reproducibility. The construct-specific detection method turned out to be less robust than the event-specific method, as the measurement results were dependent on the DNA extraction method applied. This cannot be concluded from the overall reproducibility data. When the pDNA calibrants were applied, the RSD for the method reproducibility ranged from 12 to 18% and from 13 to 19% for the construct-specific and event-specific detection methods, respectively, for GM mass fractions between 0.8 and 4.5%. Therefore, these reproducibility data alone do not allow one to judge the performance of both PCR methods.

Finally, it should be kept in mind that current GM CRMs are certified for their mass fraction of ground seeds in a mixture with ground non-GM seeds. The findings from this study provide a basis for the certification of existing GM CRMs for their DNA copy numbers using real-time PCR.

ABBREVIATIONS USED

CCQM, Consultative Committee for Amount of Substance; CRM, Certified Reference Material; CTAB, cetyl trimethyl ammonium bromide; DNA, deoxyribonucleic acid; DTCS, dye terminator cycle sequencing; gDNA, genomic DNA; GM, genetically modified; GMO, genetically modified organism(s); *hmg*, high mobility group gene; *hsp70/cryIA(b)*, junction region between the no. 1 intron sequence of the heat shock protein 70 gene and the *Bacillus thuringiensis* gene encoding CryIA(b); IRMM, Institute for Reference Materials and Measurements; *p*, probability; plant/P35S junction, junction between the integration-border region of the plant genomic sequence and the inserted sequence element originating from the cauliflower mosaic virus 35S promoter; PCR, Polymerase Chain Reaction; pDNA, plasmidic DNA; RSD, relative standard deviation; SD, standard deviation; *zSSIIb*, *Zea* starch synthase IIb gene.

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LITERATURE CITED

(1) European Commission. Commission Recommendation 2004/787/EC of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003. *Off. J. Eur. Union* **2004**, *L 348*, 18−26.

(2) European Commission. Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* **2003**, *L 268*, 1−23.

(3) Trapmann, S.; Emons, H. Reliable GMO analysis. *Anal. Bioanal. Chem.* **2005**, *381*, 72−74.

(4) Pietsch, K.; Waiblinger, H. U.; Brodmann, P.; Wurz, A. Screeningverfahren zur Identifizierung 'gentechnisch veränderter' pflanzlicher Lebensmittel. *Dtsch. Lebensm.-Rundsch.* **1997**, *2*, 35−38.

(5) Väitilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. *J. Agric. Food Chem.* **1999**, *47*, 5261−5266.

(6) European Commission. Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation. *Off. J. Eur. Union* **2004**, *L 102*, 14−25.

(7) ISO 21570. Foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—quantitative nucleic acid based methods; International Organization for Standardization: Geneva, Switzerland, 2005.

(8) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. *J. AOAC Int.* **2002**, *85*, 1119−1126.

(9) Taverniers, I.; Windels, P.; Van Bockstaele, E.; De Loose, M. Use of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products. *Eur. Food Res. Technol.* **2001**, *213*, 417−424.

(10) Mattarucchi, E.; Weighardt, F.; Barbati, C.; Querci, M.; Van den Eede, G. Development and applications of real-time PCR standards for GMO quantification based on tandem-marker plasmids. *Eur. Food Res. Technol.* **2004**, *221*, 511−519.

(11) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantification of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077−1089.

(12) Harn, C.; Knight, M.; Ramakrishnan, A.; Guan, H.; Keeling, P. L.; Wasserman, B. P. Isolation and characterization of the zSSIIa and zSSIIb starch synthase cDNA clones from maize endosperm. *Plant Mol. Biol.* **1998**, *37*, 639−649.

(13) Krech, A. B.; Wurtz, A.; Stemmer, C.; Feix, G.; Grasser, K. D. Structure of genes encoding chromosomal HMG1 proteins from maize. *Gene* **1999**, *234*, 45−50.

(14) Holck, A.; Vaitilingom, M.; Didierjean, L.; Rudi, K. 5′-nuclease PCR for quantitative event specific detection of the genetically modified MON 810 MaisGard maize. *Eur. Food Res. Technol.* **2002**, *213*, 449−454.

(15) Trapmann, S.; Conneely, P.; Contreras, M.; Corbisier, P.; Gancberg, D.; Gioria, S.; Schimmel, H.; Emons, H. *The Certification of Reference Materials of Dry-Mixed Maize Powder with Different Mass Fractions of GA21 Roundup Ready® Maize, Certified Reference Materials IRMM-414*; European Commission, IRMM, EUR Report 21203; Office for Official Publications of the European Communities: Luxembourg, 2004; 22 pp.

(16) Trapmann, S.; Le Guern, L.; Prokisch, J.; Robouch, P.; Kramer, G. N.; Schimmel, H.; Pauwels, J.; Querci, M.; Van den Eede, G.; Anklam, E. T*he Certification of Reference Materials of Dry Mixed Maize Powder with Different Mass Fractions of MON 810 Maize, Certified Reference Materials IRMM-413*; European Commission, IRMM, EUR Report 20111; Office for Official Publications of the European Communities: Luxembourg, 2001; 18 pp.

(17) Edwards, K.; Johnstone, C.; Thompson, C. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **1991**, *19*, 1349.

(18) Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989; Book 1.

(19) Poggio, L.; Rosato, M.; Chiavirino, A. M.; Naranjo, C. A. Genome size and environmental correlations in maize (*Zea mays* ssp. *mays*, Poaceae). *Ann. Bot.* **1998**, *82*, 107−115.

(20) Arumuganathan, K.; Earle, E. D. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep.* **1991**, *9*, 211−215.

(21) Guidance document. Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing; available at http://gmo-crl.jrc.it/doc/Method%20requirements.pdf.

(22) Smith, D. S.; Maxwell, P. W. Use of quantitative PCR to evaluate several methods for extracting DNA from corn flour and cornstarch. *Food Control* **2005**, *53*, 9848−9859.

(23) Peano, C.; Samson, M. C.; Palmieri, L.; Gulli, M.; Marmiroli, N. Qualitative and quantitative evaluation of the genomic DNA extracted from GMO and non-GMO foodstuffs with four different DNA extraction methods. *J. Agric. Food Chem.* **2004**, *52*, 6962−6968.

(24) Moreano, F.; Busch, U.; Engel, K.-H. Distortion of genetically modified organism quantification in processed foods: influence of particle size compositions and heat-induced DNA degradation. *J. Agric. Food Chem.* **2005**, *53*, 9971−9979.
(25) Taverniers, I.; Van Bockstaele, E.; De Loose, M. Cloned plasmid DNA fragments as calibrators for controlling GMOs: different real-time duplex quantitative PCR methods. *Anal. Bioanal. Chem.* **2004**, *378*, 1198−1207.
(26) ISO 21571. Foodstuffs—methods of analysis for the detection of genetically modified organisms and derived products—nucleic acid extraction; International Organization for Standardization: Geneva, Switzerland, 2005.
(27) Holst-Jensen, A.; Berdal, K. G. The modular analytical procedure and validation approach, and the units of measurement for genetically modified materials in foods and feeds. *J. AOAC Int.* **2004**, *87*, 927−936.

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