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Toward Metrological Traceability for DNA Fragment Ratios in GM Quantification. 1. Effect of DNA Extraction Methods on the Quantitative Determination of Bt176 Corn by Real-Time PCR

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An international CCQM-P60 pilot study involving eight national metrological institutes was organized to investigate if the quantification of genetically modified (GM) corn powder by real-time PCR was affected by the DNA extraction method applied. Four commonly used extraction methods were compared for the extraction of DNA from a GM Bt176 corn powder. The CTAB-based method yielded the highest DNA template quantity and quality. A difference in the 260 nm/230 nm absorbance ratio was observed among the different extraction methods. Real-time amplification of sequences specific for endogenous genes *zein* and *hmg* as well as transgenic sequences within the *cryIA(b)* gene and a fragment covering the junction between the transformed DNA and the plant genome were used to determine the GM percentage. The detection of the transgenic gene was affected by the quantity and quality of template used for the PCR reaction. The Bt176 percentages measured on diluted or purified templates were statistically different depending on the extraction method applied.

KEYWORDS: Feed; food; genetically modified organism; GM; measurement uncertainty; extraction method; DNA; PCR

INTRODUCTION

The European Union has elaborated legislation for genetically modified (GM) food covering preauthorization safety assessment by the European Food Safety Authority, availability of validated detection methods and reference materials, imposition of thresholds for labeling and traceability requirements, and postmarket monitoring (*1*, *2*). Labeling systems for the unintentional presence of genetically modified organisms (GMO) in food products have also been introduced in other countries such as South Korea, Japan, and Australia, but labeling of GM foods is currently not compulsory in the United States and Canada (*3*). As a consequence, the governments of several countries have announced threshold levels for the labeling of food products containing GMO varying from 0.9% in the European Union (EU) to 5% in Japan and China.

To confirm the presence of GMO and to ensure the reliability of the labeling systems, the measurement of deoxyribonucleic acid (DNA) by Polymerase Chain Reaction (PCR) has been widely used. Real-time PCR is certainly the method of choice for the quantification of DNA in appropriate measuring solutions. However, the complete analytical procedure for GM

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quantification in food and feed consists of several additional sequential steps including sample preparation, DNA extraction and purification, real-time PCR measurement and its calibration, and data evaluation, which finally provides the measurement result.

Sources of uncertainties are associated with each of these steps. The uncertainty components for the different steps are currently being evaluated at different levels by several groups (European Network for GMO Laboratories, Consultative Committee for Amount of Substance—Metrology in Chemistry). Moreover, to harmonize each individual step of the analytical procedure for the detection and quantification of GMO and derived products, a number of methods have recently been published by the European Organization for Standards (CEN) and were further implemented by the International Organization for Standardization (ISO) bodies. The International Standard (*4*) specifies how to use the standards for sampling strategies (*5*) and nucleic acid extraction (*6*), as well as qualitative (*7*) and quantitative nucleic acid analysis (*8*).

However, despite these efforts in international standardization, the minimum quality requirements that extracted DNA should meet for a quantitative real-time PCR measurement are not discussed in any detail. Indeed, only the real-time PCR modules are generally validated by assay developers and not the complete analytical procedure. Because PCR requires a high-quality DNA template defined in terms of DNA integrity and purity, the method used for the extraction of the DNA from a material is of critical importance, as has been mentioned elsewhere (*9*, *10*).

Previous investigations suggested that different extraction methods could influence the DNA quantification in food products through real-time PCR (*11*). The calibration curves obtained with DNA extracted from MON 810 corn were different depending on the extraction method used. Comparison of the DNA yield and quality following the application of several extraction methods to corn flour and cornstarch suggested that the DNA extraction efficiency had a greater influence over amplification of the target DNA, the sucrose synthase gene, than the template quality (*12*). Other studies have demonstrated that the precise quantification of GMO was affected by the degree of processing of the matrix from which genomic DNA (gDNA) was extracted (*13*). Similar influences of the GM quantification have been reported for processed corn (*14*). The similarity of the PCR efficiencies obtained for gDNA extracted from unknown samples and obtained for the DNA used for calibration has been mentioned recently as an important criterion to ensure correct PCR quantification (*15*).

Some authors have therefore proposed a so-called modular approach to facilitate the validation of the GM measurement procedure (*16*). This pragmatic approach implies that each step in the analytical procedure can be decoupled provided that each step fulfills certain quality criteria. The advantage of such an approach resides in the fact that separately validated methods, for example, for DNA extraction or real-time PCR, could be combined. Moreover, if the real-time PCR measurement is not influenced by the type of extraction method applied, the validation of the real-time PCR measurement can be performed on DNA extracted with any method and from any type of matrix, but scientific evidence for this approach has still to be provided.

The aim of the present study was to investigate if the DNA extraction methods commonly applied prior to real-time PCR analysis have an effect on the quantification of Bt176 corn. The measurement uncertainties for the DNA extraction and the GM quantification step have been estimated using a Bt176 corn certified reference material (CRM) as model for the study.

MATERIALS AND METHODS

Study Setup. Eight laboratories participated in the pilot study, which was conducted through the Consultative Committee for Amount of Substance (CCQM) Working Group on Bioanalysis. The organizing laboratory (IRMM, Belgium) prepared and dispatched the required materials, the detailed protocols, and data template forms to the other laboratories, which were asked to perform the analyses and to send the data back to IRMM. IRMM then evaluated the data and performed the statistical analysis. The materials provided to each laboratory consisted of one unknown corn powder sample (two bottles, A and B, of 1 g), two vials of dried gDNA prepared from 100% Bt176 corn for calibration, one bottle of nuclease-free water (Promega Benelux, Leiden, The Netherlands), vials with lyophilized primers and probes, and either the DNeasy Plant Mini Kit (Qiagen Benelux B.V.) or the Wizard genomic DNA purification kit (Promega Benelux), or both, depending on the agreed extraction method(s) assigned to that laboratory. The primers and probes were reconstituted by participating laboratories in nuclease-free water and stored at −20 °C. The cetyltrimethylammonium bromide (CTAB) buffers or chemicals required for their preparation, RNase A, proteinase K, chloroform (>99% purity), absolute ethanol, isopropanol, and reagents used in gel electrophoresis were from suppliers of choice of a participant laboratory.

Powder Material. The unknown blind sample analyzed was a CRM powder (ERM-BF411e, IRMM, Belgium) certified for its mass fraction of Bt176 corn (20.0 ± 1.1 g/kg) (*17*). Two CRM bottles were sent to every laboratory.

Genomic DNA Isolation. Four methods, routinely used by many laboratories, were employed to extract DNA from the sample. Every laboratory was asked to use at least two different extraction methods, of which one was the CTAB method as that method is not dependent on a kit's components or covered by patent rights. For each extraction method, the unknown sample (Bt176 corn) was extracted in triplicate on day 1 (from bottle A) and again on day 2 (using bottle B).

(i) The CTAB (*18*) DNA extraction method was performed by adding 300 μL of nuclease-free water, 700 μL of CTAB extraction buffer [CTAB, $\rho = 20$ g/L, 1.4 M NaCl, 0.1 M Tris-HCl, and 20 mM EDTA, pH 8], and 5 μL of RNase A solution (100 mg/mL) to 100 mg of each sample and incubating the mixture at 65 °C during 15 min. After the addition of 20 μL of proteinase K (20 mg/mL), the lysate was further incubated for another 15 min at 65 °C. The mixture was centrifuged at 12000*g* for 10 min. The supernatant was transferred to a new tube and mixed with 500 μL of chloroform. After 10 min of centrifugation, 700 μL of the aqueous phase was transferred to a new tube, mixed with the same volume of chloroform, and centrifuged for 5 min at 12000*g*. The aqueous phase was removed and mixed with a double volume of CTAB precipitation buffer (CTAB, $\rho = 5$ g/L, 40 mM NaCl). Following 1 h of incubation at room temperature, the DNA was pelleted at 12000*g* for 15 min. The supernatant was discarded, and the DNA was resuspended in 400 μL of 1.2 M NaCl. Following the addition of 400 μL of chloroform, mixing, and phase separation by centrifugation for 10 min, the aqueous phase was transferred to a new tube and gently mixed with a double volume of ice-cold absolute ethanol. The solution was incubated for at least 20 min at −20 °C and then centrifuged for 15 min at 12000*g*. The DNA pellet was washed with 500 μL of 70% v/v ethanol, centrifuged, and air-dried. One hundred microliters of nuclease-free water (preheated at 65 °C) was added, and the DNA extracts were kept overnight at 4 °C to allow complete rehydration.

(ii) The DNeasy Plant Mini Kit (Qiagen Benelux B.V., catalog no. 69104) was used according to the manufacturer's instructions with slight modifications. In preliminary experiments, a sample intake of 100 mg was found to be appropriate for this method, yielding good-quality DNA. A volume of 400 μL of buffer AP1 and 4 μL of RNase A solution (100 mg/mL) were added to each sample (100 mg). The lysis mixture was incubated for 1 h at 65 °C, mixing the lysate every 10−15 min by inverting the tube. A volume of 130 μL of buffer AP2 was added to the lysate, mixed, and incubated for 5 min on ice. The lysate was then centrifuged for 5 min at 12000*g*, and the supernatant was applied to a QIAshredder Mini Spin Column placed in a 2 mL collection tube. Following 2 min of centrifugation at 12000*g*, the flow-through fraction was transferred to a new tube, and 1.5 volumes of buffer AP3/E was

Table 1. Oligonucleotides Used in This Study

name	orientation	sequence	amplicon length (bp)	ref
Hmg	forward	5′-TTG GAC TAG AAA TCT CGT GCT GA-3′	79	*8*
	reverse	5′-GCT ACA TAG GGA GCC TTG TCC T-3′		
	probe	5′-(FAM)-CAA TCC ACA CAA ACG CAC GCG TA-(TAMRA)-3′		
cry1A(b)	forward	5′-CCC ATC GAC ATC AGC CTG AGC-3′	129	*8*
	reverse	5′-CAG GAA GGC GTC CCA CTG GC-3′		
	probe	5′-(FAM)-ATG TCC ACC AGG CCC AGC ACG-(TAMRA)-3′		
zein	forward	5′-GCC ATT GGG TAC CAT GAA CC-3′	104	*20*
	reverse	5′-AGG CCA ACA GTT GCT GCA G-3′		
	probe	5′-(FAM)-AGC TTG ATG GCG TGT CCG TCC CT-(TAMRA)-3′		
Bt176-plant junction	forward	5′-CTT CAG CCT GCC GGT ACT G-3′	85	*19*
	reverse	5′-CAT TGA TGG CGT GCA TCA AT-3′		
	probe	5′-(FAM)-CGT CAC CGA GAT CTG ATG TTC TCT CCT CC-(TAMRA)-3′		

added and mixed by pipetting; 650 μL of the mixture was then applied to a DNeasy Mini Spin Column placed in a collection tube. The column was centrifuged for 1 min at 6000*g*, the flow-through was discarded, and the last step was repeated with the remaining lysate. The DNeasy Mini Spin Column was then placed in a new 2 mL tube, and 500 μL of buffer AW was added. After 2 min of centrifugation at 12000*g*, the column was placed in a new 1.5 mL tube, and 100 μL of preheated nuclease-free water (65 °C) was applied to the DNeasy membrane. After 5 min of incubation at room temperature, the DNA was eluted from the silica spin column by centrifugation for 1 min at 6000*g*.

(iii) The protocol for the isolation of gDNA from plant tissue using the Wizard Genomic DNA Purification Kit (Promega Benelux catalog no. A1120) was followed according to the manufacturer's instructions with the only modification of using DNase-free water to rehydrate the DNA pellet. The sample intake was determined in preliminary experiments, revealing that a maximum of 20 mg of corn powder could be used per extraction; higher masses (33, 50, or 100 mg) yielded DNA extracts of lower quality containing PCR inhibitors. As a consequence, five subsamples of 20 mg of powder were extracted per sample, and the DNA was combined into one tube at the end. A volume of 600 μL of nuclei lysis solution was added to each subsample, mixed by pipetting, and incubated at 65 °C for 15 min; 3 μL of RNase A solution (100 mg/mL) was added, and the lysis mixture was incubated at 37 °C for 15 min. Once the mixture had cooled to room temperature, 200 μL of Protein Precipitation Solution was added, and the solution was mixed by vortexing. After centrifugation at 12000*g* for 10 min, the supernatant was transferred to a new tube containing 600 μL of isopropanol at room temperature. The solution was mixed and centrifuged at 12000*g* for 1 min, and the supernatant was decanted. The DNA pellet was washed with 600 μL of 70% v/v ethanol and centrifuged, and the ethanol was discarded. The pellet was air-dried at room temperature, then resuspended in 30 μL of nuclease-free water, and kept overnight at 4 °C to allow complete rehydration of the DNA; the extracts from five tubes were combined into one tube on the following day.

(iv) The Nippon Gene GM Quicker protocol was followed according to the manufacturer's recommendations (kindly provided by Diagenode N.V., Liège, Belgium). A volume of 600 μL of GE1 buffer and 4 μL of RNase A (100 mg/mL) were added to 100 mg of sample and mixed by vortexing for 30 s. Cell lysis was achieved by incubation at room temperature for 5 min, and then 75 μL of GE2 buffer was added to the tube, which was inverted several times before being incubated on ice for 5 min. After 10 min of centrifugation at 12000*g* at 4 °C, 400 μL of the supernatant was carefully transferred to a new 1.5 mL tube, and 50 μL of GB3 buffer and 200 μL of absolute ethanol were consecutively added. After mixing by inverting the tube until a clear solution was obtained, the mixture was transferred to a spin column and centrifuged for 30 s at 4 °C at 12000*g*. The flow-through was removed, and 600 μL of GW washing buffer was added to the spin column, which was then centrifuged for 30 s at 4 °C at 12000*g*. The purified gDNA was eluted from the column by adding 50 μL of DNase-free water and centrifugation for 1 min at 12000*g* at 4 °C.

Qualitative Assessment of DNA Integrity. DNA extracts were analyzed on 0.8% agarose (Invitrogen) gels. The gels contained 0.5 μg/mL ethidium bromide and were run in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA). Digital images of the gels were viewed and captured using different imaging systems.

DNA Quantification. The extracted DNA was quantified using the Picogreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR) according to the kit instructions. The purity of the DNA in the solution was analyzed spectrophotometrically at 230, 260, and 280 nm using a UV−vis spectrophotometer (such as UV-1700 Shimadzu, NanoDrop ND-1000, or Biophotometer Eppendorf), and the ratios of the absorbance at 260 and 280 nm ($Abs_{260/280}$) and of the absorbance at 260 and 230 nm ($Abs_{260/230}$) were calculated to provide an estimation of the quality of the extracted DNA. Three dilutions of each DNA preparation—10, 5, and 1.25 ng/μL—were prepared for real-time PCR analysis.

Quantification of the Bt176 Corn Event. The quantification of the transgenic target was performed using two published detection methods (**Table 1**): (1) a construct-specific method targeting a 129 bp fragment of the *cry1A(b)* gene and a 79 bp fragment of the *hmg* gene used as normalizer (*8*) and (2) an event-specific method targeting an 85 bp fragment of the Bt176-plant 5′-junction region (further referred to as the Bt176-plant junction) (*19*) combined with a 104 bp fragment of the *zein* gene (*20*). In brief, the final concentration in the PCR reaction of relevant primers was 300 nM in both methods, whereas the concentration of probe was 160 and 200 nM for the first and second methods, respectively. TaqMan Universal PCR analyses were carried out according to the manufacturer's instructions (Applied Biosystems, ABI, Foster City, CA) with universal thermal profile consisting of an initial denaturation step at 95 °C for 10 min, followed by 45 amplification cycles of 15 s at 95 °C, and 1 min at 60 °C. Five microliters of template DNA or nuclease-free water was used in 50 μL of final reaction volume per PCR well. Each PCR measurement was repeated in triplicate on two different days.

Calibration Curves. The calibrant used in the study consisted of dried gDNA extracted by the CTAB method from the bulk ground seed powder of Bt176 corn used in the gravimetrical preparation of the reference material ERM-BF411e. Five micrograms of vacuum-dried gDNA was provided to the participating laboratories and dissolved overnight in 100 μL of DNase-free water to obtain a solution of 50 ng/μL. The concentration was confirmed by each laboratory, and the DNA was further diluted to 10 ng/μL. Dilution series were prepared from gDNA in nuclease-free water ranging from 10 to 0.005 ng/μL corresponding to 100−0.05% (m/m) Bt176. The calibration series were kept at 4 °C and mixed before use. Real-time PCR analyses were performed as described above.

Statistical Analysis. The GM percentage for the unknown sample was calculated by extrapolation of the Ct values reported by each laboratory with their standard curves. No Ct values were excluded from the study unless a technical reason was reported by a laboratory. Statistical analysis was performed using the Statistica 7.0 software (Statsoft, Tulsa, OK). The unimodality and normal distribution probability of the GM percentage were confirmed, and therefore no log-normal transformation of the data was needed. The significance of parameters was analyzed by an analysis of the variance. The sigma restricted parametrization for factorial designs was applied. The existence of a relationship between the tested parameters was evaluated by performing a factorial analysis of the variance. Relative standard

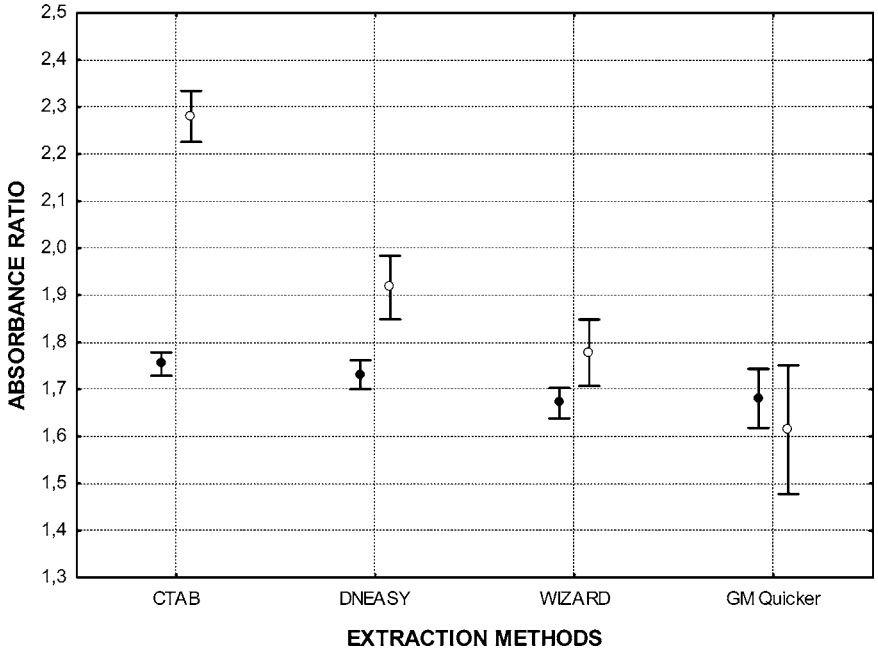

Figure 1. Absorbance ratios [$Abs_{260/280}$ (●) and $Abs_{260/230}$ (○)] of gDNA extracted by various methods. The vertical bars denote 95% confidence intervals. The mean values are derived from 52 observations for the CTAB method, 36 observations for the DNeasy method, 30 observations for the Wizard method, and 12 observations for the GM Quicker method.

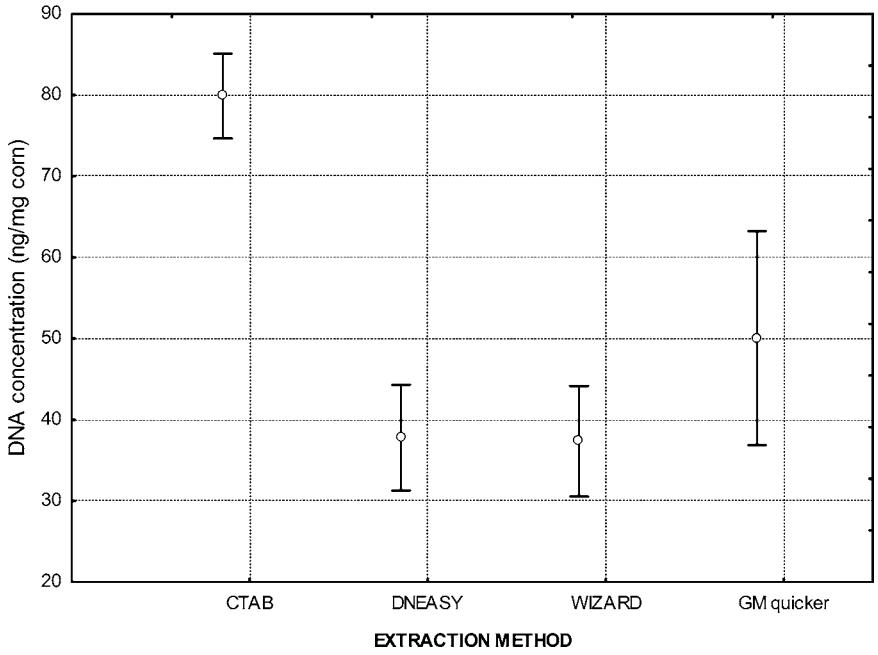

Figure 2. Concentration of DNA prepared by various extraction methods. The vertical bars denote 95% confidence intervals. The mean values are derived from 52 observations for the CTAB method, 36 observations for the DNeasy method, 30 observations for the Wizard method, and 12 observations for the GM Quicker method.

deviation (RSD) of repeatability was calculated by ANOVA as being the square of the within-laboratory mean of the square divided by the average of the study, whereas the relative intermediate precision was calculated as the square root of the difference of between laboratories and the within-laboratory variation divided by the number of duplicates and divided by the average of the measurements.

RESULTS AND DISCUSSION

Setup of the Study. The eight laboratories participating in this study were National Metrology Institutes with previous experience in real-time PCR quantification. The goal of the study was to determine if the quantification of a GM event by real-time PCR delivered the same results independently of the extraction method applied on ground corn seed material. To minimize the sources of potential variability (*21*) using real-time PCR methods, several critical measurement parameters were kept identical among the participants. The measurements were performed using the same PCR platform (ABI7700 or ABI7900), the same TaqMan chemistry, and the same real-time PCR methods. All consumables used in PCR analyses including primers and probes were derived from the same batch, and the calibrant used was identical. The extraction kits used in this study had also the same lot number. Only the TaqMan Universal PCR MasterMix (Applied Biosystems) was purchased indepen-

dently by participating laboratories as it could not be dispatched at room temperature as the rest of the materials and consumables.

The analyzed sample was a very well characterized CRM corn powder used as a model matrix, from which gDNA was extracted by using the extraction methods commonly applied by GM analysis laboratories. The CRM corn powder represents a relatively easy matrix compared to processed food samples or fresh plant materials containing either shorter DNA fragments or several coextracted compounds that can affect the efficiency of the PCR reaction (*13*). As the corn sample analyzed was not calibrated by a PCR measurement procedure but gravimetrically prepared using the GM corn powder from which it was derived (*17*), the GM percentages were unaffected by parameters such as DNA extractability from corn embryo and endosperm (*22*, *23*) or from the parental origin of the Bt176 event. Consequently, the trueness of the measurements of this study could be established.

Qualitative and Quantitative Analysis of the Extracted DNA. The $Abs_{260/280}$ and $Abs_{260/230}$ ratios were calculated to estimate the quality of the DNA extracted by the different laboratories using the different extraction methods (**Figure 1**). The $Abs_{260/280}$ ratios were all slightly below 1.8, indicating that the preparations were mainly enriched in DNA but that remaining traces of proteins or aromatic substances could still be present. The $Abs_{260/280}$ ratios of the DNA preparations obtained using the four extraction methods were not statistically different from each other.

The $Abs_{260/230}$ ratio of the DNA preparations varied depending on the extraction method applied. The DNA preparations obtained with the Nippon Gene GM Quicker had the lowest $Abs_{260/230}$ ratio of 1.62 but were also the less contaminated by RNA, which is known to strongly absorb light at 260 nm. Low $Abs_{260/230}$ ratios may indicate the presence of carbohydrates that are known to interfere in the PCR (*24*). Both the CTAB method and the DNeasy method delivered the purest DNA extracts with ratios of 2.28 ± 0.18 and 1.94 ± 0.21, respectively.

The quantity of DNA that could be extracted from a sample also varied depending on the extraction method applied (**Figure 2**). The highest yields were obtained with the CTAB method, whereas the DNeasy, Wizard, and Nippon Gene GM Quicker methods (both based on silica membrane based method) yielded lower but similar DNA amounts. The difference in efficiency of extraction of corn kernel DNA confirmed earlier reports (*10*). The amount of DNA extracted with any method was, however, sufficient for PCR amplification even if it corresponded only to a small fraction of the total DNA content of corn. To estimate the total mass of DNA in the powder, the DNA in the sample was hydrolyzed by perchloric acid (*25*) and the DNA was quantified using diphenylamine (*26*). The CTAB method extracted only 25% (m/m) of the DNA content present in the powder, but as the amount of transgenic genes is normalized by the reference genes, the total amount of DNA extracted should have no impact on the quantification.

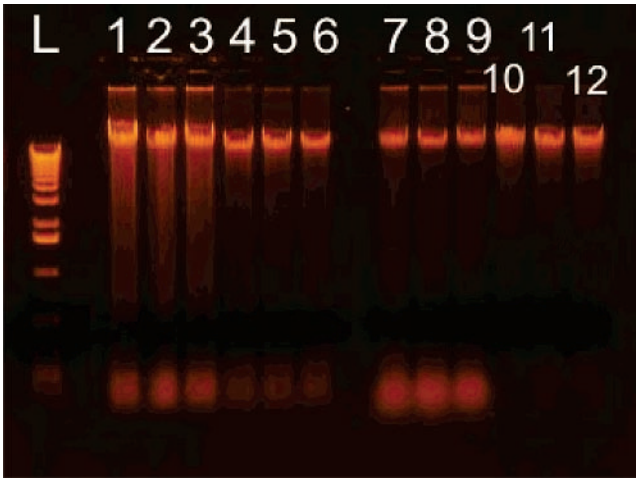

Figure 3. Agarose gel electrophoresis of gDNA extracted from the corn powder [typical example of gel loaded with three DNA extracts (5 μL each) per extraction method]: lane L, 1 kb DNA ladder; lanes 1–3, CTAB; lanes 4–6, DNeasy; lanes 7–9, Wizard; lanes 10–12, GM Quicker.

The extracted DNA was tested for DNA degradation by determining the range of fragment size of the DNA isolated by using the four extraction methods (**Figure 3**). DNA was size-sorted using gel electrophoresis in agarose gels, stained with ethidium bromide, and compared to a DNA marker. DNA fragments above 12 kb were prominent in the DNA prepared by each method, suggesting that the DNA was not excessively sheared and was suitable for amplification. Traces of contaminating RNA molecules could be observed in the electrophoresis gel for the DNA preparations extracted by all extraction methods except the Nippon Gene GM Quicker method, indicating an incomplete digestion of the RNA by RNase A.

Real-Time Quantitative PCR for the Bt176 Event. The GM content measured by the different laboratories is reported separately for the construct-specific PCR method (**Table 2**) and for the event-specific PCR method (**Table 3**). All laboratories applied the same CTAB extraction method and one or more of

Table 2. Calculated GM Percentage for the Construct-Specific *cry1A(b)*/*hmg* Quantification System on DNA Extracted by the Different Methods[a]

	CTAB			DNeasy			Wizard			GM Quicker		
lab	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL
1	1.30	1.30	1.87	1.41	1.47	1.83	1.62	1.78	2.23	1.78	1.24	1.63
2	1.92	1.73	1.75	2.07	1.83	1.81	*	*	*	*	*	*
3	2.23	2.18	1.85	1.94	1.87	1.93	*	*	*	1.75	1.80	1.61
4	1.35	1.59	1.76	*	*	*	1.53	1.49	2.09	*	*	*
5	1.93	2.12	2.39	1.58	1.54	1.87	*	*	*	*	*	*
6	1.63	1.85	2.40	*	*	*	1.82	2.01	2.29	*	*	*
7	1.40	1.46	1.68	*	*	*	1.67	1.83	2.20	*	*	*
8	1.17	1.27	1.68	*	*	*	1.67	1.76	1.91	*	*	*
N	8	8	8	4	4	4	5	5	5	2	2	2
av	1.61	1.69	1.92	1.75	1.67	1.86	1.66	1.77	2.14	1.77	1.52	1.62
sd	0.37	0.35	0.30	0.31	0.20	0.05	0.10	0.19	0.15	0.02	0.39	0.02
RSD	23.2	20.6	15.6	17.5	12.2	2.74	6.27	10.6	7.08	0.87	25.9	0.99

[a] Each value corresponds to the average of six extracts. *, not measured.

Table 3. Calculated GM Percentage for the *Bt176-Plant Junction/zein* Event-Specific Quantification System[a]

	CTAB			DNeasy			Wizard			GM Quicker		
lab	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL
1	1.04	1.26	1.90	1.46	1.71	1.92	2.00	2.04	1.94	1.76	1.43	1.88
2	1.28	1.24	1.46	1.15	1.32	1.61	*	*	*	*	*	*
3	1.43	1.63	2.15	1.67	1.83	2.16	*	*	*	2.16	2.23	2.40
4	1.45	1.42	2.10	*	*	*	1.86	1.79	2.23	*	*	*
N	4	4	4	3	3	3	2	2	2	2	2	2
av	1.30	1.39	1.90	1.42	1.62	1.90	1.93	1.91	2.09	1.96	1.83	2.14
sd	0.19	0.18	0.31	0.26	0.27	0.28	0.10	0.18	0.21	0.28	0.57	0.37
RSD	14.6	13.3	16.4	18.4	16.6	14.6	5.04	9.45	9.97	14.1	30.9	17.2

[a] Each value corresponds to the average of six extracts. *, not measured.

the three other extraction methods. All laboratories tested the construct-specific detection method, whereas four of the eight laboratories tested also the event-specific detection method, generating in total 402 data points for the construct-specific detection method and 258 data points for the event-specific method.

The PCR analyses were performed on two different days on separate plates and as no significant effect ($p > 0.05$) from 1 day to another were observed within the same laboratory, values obtained on day 1 have been pooled with the values reported for day 2. Main effects ANOVA and factorial ANOVA were performed on the totality of the results to identify the parameters affecting the measured GM content. Both detection methods were performed on DNA preparations at three predefined template concentrations in the PCR from 10 to 1.25 ng/μL. The concentration of template used in the PCR reaction had a significant impact ($p < 0.001$) on the trueness of the measurement results. When testing DNA at 10 and 5 ng/μL (50 and 25 ng of DNA template per well, respectively), all laboratories underestimated the Bt176 content, giving general averages of 1.63 $\pm$ 0.31% (m/m) and 1.69 $\pm$ 0.29% (m/m) for DNA concentrations of 10 and 5 ng/μL, respectively. The underestimation was independent of the detection method applied and the extraction procedure used. However, when the DNA template was diluted in DNase-free water to 1.25 ng/μL, the average Bt176 content reported increased to 2.05 $\pm$ 0.45% (m/m) Bt176, which was close to the certified GM Bt176 value [2.00 $\pm$ 0.11% (m/m)].

The underestimation of the GM content for the more concentrated DNA samples was investigated by carefully evaluating the linearity of the calibration curves. The average PCR efficiencies of the calibration curves for the GM and reference targets for both the construct- and event-specific methods were all within acceptance criteria as defined for control chart warning limits (*27*) and were statistically not different from each other ($p > 0.05$) (**Table 4**). However, when the Ct values were plotted as a function of the template concentration, it was noticed that the calibration curve for the amplification of the *cryIA(b)* target was positively skewed at the highest template concentrations (**Figure 4**, solid circles). This effect was not observed for the endogenous *hmg* gene (solid squares). To investigate if this effect was caused by the presence of PCR inhibitors, the following test was done. The gDNA extracted by laboratory 1 was further purified by anion-exchange chromatography using a gravity-flow column Genomic-Tip 20 (Qiagen, Hilden, Germany). During that subsequent procedure, DNA fragments bound to the column by electrostatic interactions between the negatively charged phosphate groups of the nucleic acid backbone and the positively charged anion-exchange resin. Subsequent washing steps removed potential impurities, and the DNA was eluted with a high-salt buffer, desalted, and concentrated by a final isopropanol precipitation step. The gDNA purified on the Tip 20 columns was further tested by real-time PCR (**Figure 4**, open symbols), and a perfect linearity was obtained up to 10 ng/μL DNA. DNA from the test sample extracted by both the CTAB and Wizard methods was further purified using the Genomic-Tip 20 column, and the Bt176 content was determined by the construct-specific method using

Table 4. Calculated PCR Efficiencies Expressed in Percentage for the *cry1A(b)/hmg* and *Bt176-Plant Junction/zein* Quantification Systems[a]

lab	*hmg* (%)	*cry1A(b)* (%)	*zein* (%)	*Bt176-junct* (%)
1	117	100	112	83
	118	111	110	82
	114	95	109	98
	114	92	107	104
2	99	125	111	93
	115	110	114	88
3	98	118	98	78
	100	107	101	85
	104	94	98	85
4	114	113	113	85
	119	111	113	100
5	103	103	110	109
6	114	107	107	90
7	109	94	109	96
8	119	112	*	*
av	110	107	108	91
s	7.65	9.69	5.34	9.09
CV (%)	6.96	9.09	4.95	9.97

[a] Each line corresponds to an independent calibration curve. *, not measured.

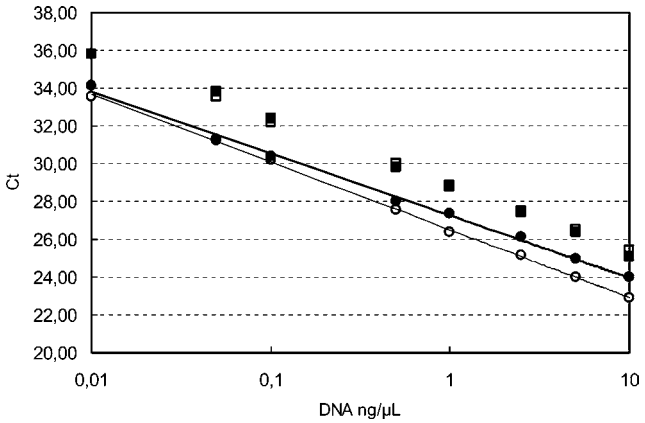

Figure 4. Calibration curves for the *cry1A(b)* (circles) and *hmg* (squares) genes on Bt176 DNA extracted by the CTAB method (solid symbols) or additionally purified on Genomic P20 columns (open symbols).

Table 5. Bt176 Content Analyzed by the Construct-Specific Method on DNA Extracts Purified by the CTAB and Wizard Methods and by the CTAB and Wizard Methods Followed by an Additional Purification Step Using Genomic P20 Anion-Exchange Columns Undertaken by Laboratory 1[a]

	Bt176 % (m/m)		
	10 ng DNA/μL	5 ng DNA/μL	1.25 ng DNA/μL
CTAB	1.62 ± 0.35	1.71 ± 0.33	2.04 ± 0.45
CTAB + G20	2.15 ± 0.31	2.15 ± 0.18	2.32 ± 0.31
Wizard	1.75 ± 0.22	1.87 ± 0.30	2.46 ± 0.77
Wizard + G20	1.86 ± 0.25	2.06 ± 0.07	2.20 ± 0.08

[a] Values represent the mean values of triplicate measurements on two independent DNA extracts ± 2*s*.

the G20-purified gDNA as a calibrant (**Table 5**). As expected, the *cry1A(b)* calibration curve was linear and the calculated Bt176 content corresponded to the certified value and was independent of the initial template concentration. We could therefore assume that the presence of inhibitors (*28*) such as polysaccharides or polyphenols was influencing the amplification of *cry1A(b)* targets in insufficiently purified DNA. As the determination of the number of *cry1A(b)* DNA copies was more affected by the suspected inhibitors than the determination of the number of *hmg* copies (**Figure 4**), the calculated amount of Bt176 in extracts having a lower quality of DNA was reduced. The influence of coextracted impurities could, however, be removed by sufficient dilution of the extracts. This observation also indicates that inhibition of the PCR was not the same for the amplification of the *cry1A(b)* gene compared to the *hmg* gene. In other words, the robustness of the real-time PCR methods was not equally affected by the presence of inhibitors.

Effect of the Extraction Method on Bt176 Quantification. Main effects ANOVA and factorial ANOVA were performed on the diluted DNA template (1.25 ng/μL in the PCR) for each combination of DNA extraction method and real-time PCR detection method. The standard deviation of repeatability achieved by each laboratory could be evaluated because measurements were performed on two distinct days. As no significant effect ($p > 0.05$) of the day was observed, the later statistical analyses were performed on the pooled data points from both days. The study revealed also no significant impact ($p > 0.05$) of the detection method on the Bt176 content. Indeed, the average Bt176 content from 114 construct-specific measurements and from 66 event-specific measurements gave 1.94 ± 0.55% (m/m) and 1.98 ± 0.43% (m/m), respectively. It can be concluded that both detection methods gave identical quantification results, which were statistically identical to the certified value of 2.00 ± 0.11% (m/m).

Interestingly, a significant impact ($p < 0.01$) of some extraction methods on the measured Bt176 content was observed (**Figure 5**). The impact of the extraction method was independent of the detection method applied, except for the Nippon Gene kit. The latter effect should not be overemphasized because this extraction method was applied by only two laboratories. Statistical analysis of the data (excluding the Nippon Gene extraction data) confirmed the significant impact ($p < 0.007$) of the extraction methods on the measured Bt176 content. The CTAB and DNeasy methods gave similar GM content results; however, higher Bt176 percentages were obtained using the Wizard protocol. This significant effect ($p < 0.007$) of the extraction method on the quantification of Bt176 could be concluded, because the number of data was large and most parameters, potentially affecting the precision of the real-time PCR, had been minimized in this study. The data confirmed in a quantitative way previous observations made by other groups (*11*) and were confirmed on another corn model (part 2 of this series). Despite this impact of the DNA extraction method on the quantitative determination of Bt176 by real-time PCR, the differences between the measured Bt176 contents can be considered as relatively small, being 1.86 ± 0.27% (m/m), 1.92 ± 0.44% (m/m), and 2.17 ± 0.48% (m/m), respectively, for the DNeasy ($n = 27$), the CTAB ($n = 48$), and the Wizard methods ($n = 27$), respectively. The relative standard deviation of PCR repeatability in this particular study was not affected by the extraction method applied and was better than 25%. The relative intermediate precision of the PCR measurements of all pooled data fluctuated between 5.7 and 15.6% (**Table 6**).

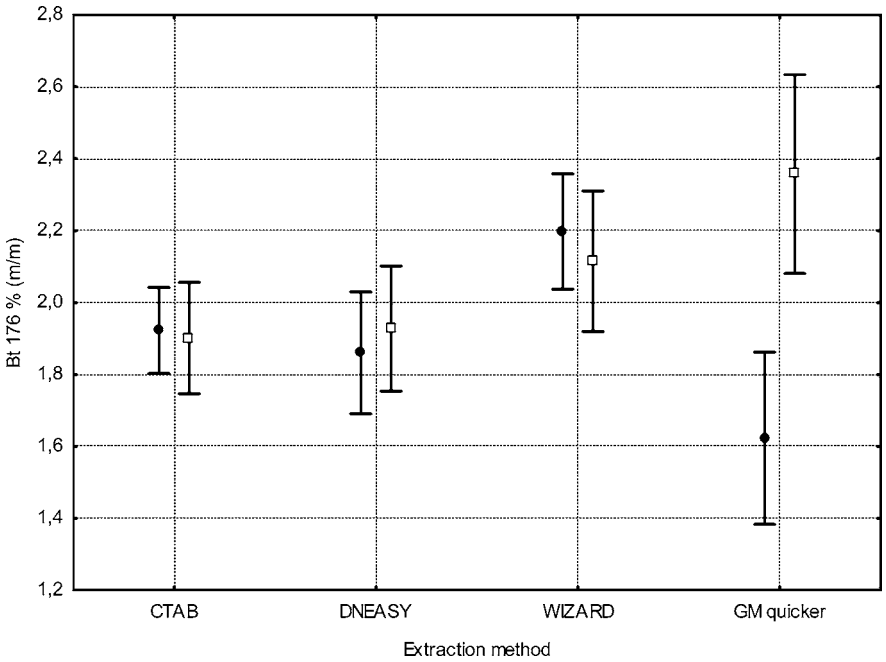

Figure 5. Effect of extraction method on Bt176 quantification. The values are presented as the least-squares means for the *cry1A(b)*/*hmg* (●) and *Bt176-plant junction*/*zein* (□) detection methods, respectively. Vertical bars denote 95% confidence intervals.

Table 6. Method Repeatability and Intermediate Precision of the Bt176 GM Content on DNA Extracted by Different DNA Extraction Methods[a]

	CTAB	DNeasy	Wizard	GM Quicker
data sets	71	42	38	20
method repeatability (%)	20.4	17.1	14.6	23.7
intermediate precision (%)	12.6	5.7	*	11.5

[a] *, MS within > MS between.

This study clearly demonstrated that the different extraction methods provided DNA preparations that strongly differed in their $Abs_{260/230}$ ratios. The CTAB method gave the highest extraction yield and the best DNA quality in terms of $Abs_{260/230}$ and $Abs_{260/280}$ ratios. However, additional purification on a P20 column or dilution of the DNA template was required to obtain linear calibration curves for the *cryIA(b)* or the Bt176-plant gDNA junction targets. The robustness of the PCR also varied depending on the target that was amplified. Most GM laboratories favor the use of extraction kits as the extraction step is relatively fast (*29*), but for some complex matrices extensive optimization of the extraction procedure is necessary (*30*). Inhibition tests using either internal controls (*31*) or evaluation of the linearity of the calibration curves should be performed to qualify the extracted DNA for real-time PCR amplification. The accuracy of the GM measurement depends significantly on the quality of the extracted DNA, which is amplified in the real-time PCR reaction. Taking those considerations into account, the study also demonstrated the ability of some National Metrology Institutes to perform accurate GM determinations.

ABBREVIATIONS USED

CCQM, Consultative Committee for Amount of Substance; CRM, Certified Reference Material; CTAB, cetyl trimethyl ammonium bromide; DNA, deoxyribonucleic acid; gDNA, genomic DNA; GM, genetically modified; GMO, genetically modified organism(s); IRMM, Institute for Reference Materials and Measurements; PCR, Polymerase Chain Reaction; NMI; National Metrology Institute; MS, mean of the squares; sd, standard deviation.

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