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ARTICLE

Finding the Joker among the Maize Endogenous Reference Genes for Genetically Modified Organism (GMO) Detection

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The comparison of five real-time polymerase chain reaction (PCR) methods targeted at maize (*Zea mays*) endogenous sequences is reported. PCR targets were the alcohol dehydrogenase (*adh*) gene for three methods and high-mobility group (*hmg*) gene for the other two. The five real-time PCR methods have been checked under repeatability conditions at several dilution levels on both pooled DNA template from several genetically modified (GM) maize certified reference materials (CRMs) and single CRM DNA extracts. Slopes and R^2 coefficients of all of the curves obtained from the adopted regression model were compared within the same method and among all of the five methods, and the limit of detection and limit of quantitation were analyzed for each PCR system. Furthermore, method equivalency was evaluated on the basis of the ability to estimate the target haploid genome copy number at each concentration level. Results indicated that, among the five methods tested, one of the hmg-targeted PCR systems can be considered equivalent to the others but shows the best regression parameters and a higher repeteability along the dilution range. Thereby, it is proposed as a valid module to be coupled to different event-specific real-time PCR for maize genetically modified organism (GMO) quantitation. The resulting practicability improvement on the analytical control of GMOs is discussed.

KEYWORDS: GMO; real-time PCR; endogenous gene; maize; *Zea mays*; validation

INTRODUCTION

European Union (EU) legislation on genetically modified (GM) food and feed, namely, regulation EC 1829/2003, establishes that, to place GM feed and food on the market, an authorization must be granted; the authorization procedure requires the applicant to submit a detailed dossier to the National Competent Authority that informs and passes along all of the application documents to the European Food Safety Authority (EFSA). The application contains relevant information about the transformation event and its safety. For authorized products, traceability and labeling provisions set up by regulation EC 1830/2003 (*1*) and 1829/2003 (*2*), respectively, introduced the obligation to provide information on the presence of a genetically modified organism (GMO) or a product consisting, containing, or derived from a GMO along the whole food and feed supply chain. Exemption from labeling is established in the case where no more than 0.9% of each ingredient is of GM origin, provided that the GM content is adventitious or technically unavoidable.

The enforcement of traceability and labeling provisions requires that each transformation event is identified and quantified with respect to the relative ingredient; thus, detection methods must be event-specific and quantitative. DNA-based methods are perfectly fit for this purpose, because DNA sequences at the junction between the genomic insertion locus and the transgenic DNA can be exploited to attain transformation event specificity; furthermore, DNA targets offer a number of advantages for quantification purposes over other types of analyte, such as proteins (*3*).

Polymerase chain reaction (PCR) is a highly specific and sensitive methodology for detecting GMOs. Despite recent advances in the molecular technique for detecting GMOs (*4*,*5*), real-time PCR is still considered the most reliable and straightforward method for quantitative assays (*6*). For quantification purposes, the amount of GM event-specific DNA sequences is related to an endogenous reference gene distinctive of the specific ingredient. The GM ratio is obtained by comparing the quantitative results of two different real-time PCRs: a transformation event-specific one and a taxon-specific one. The quantification of the GM event in the specific ingredient of the sample can be achieved by either direct comparison of the Ct (threshold cycle, i.e., the cycle number that corresponds to the first detectable signal above the baseline) of the two reactions (ΔCt method) or using copy-number-based standard curves to determine absolute copy numbers, which can then be compared (standard curve method). In both cases, amplification of a reference gene target with a stable known and low copy number (preferably 1 copy per haploid genome) and with no allelic variation among cultivars in the same species is required (*7*−*10*). Amplification of a taxon reference gene is not only the comparator for the GMO-specific amplification but, if

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Table 1. Composition of Maize Powder Described in the Certification Report of IRMM

CRM	acronym[a]	GM variety	non-GM variety	produced by
BT11	CRM1	NX 3707 BT11	Pelican	Syngenta Seeds SAS (Nérac, France)
BT176	CRM2	GaronaBT-176	Bahia	Syngenta Seeds SAS (Nérac, France)
GA21	CRM3	RX740RR	RX670	Monsanto (St. Louis, MO)
MON810	CRM4	DK 513	DK 512	RAGT Semences (Rodez, France)
MON863	CRM5	TP5504-TD	RX670	Monsanto (St. Louis, MO)
MON810 × MON863	CRM6	TP6705-BG	RX670	Monsanto (St. Louis, MO)
NK603	CRM7	DKC 57-40	RX670	Monsanto (St. Louis, MO)

[a] Acronym used in the text.

properly and correctly performed, can provide plenty of information on the quality and quantity of the extracted DNA, because the efficiency and the Ct of the reaction are often affected by impurity and degradation of target DNA. Therefore, in routine laboratories, the so-called "monitor run" or "inhibition run", targeting reference genes of botanical species supposed to be present in the sample and carried out on the extracted DNA at different dilutions, can be a very useful preliminary step in the analytical workflow, although additional inhibition controls may be necessary (*11*).

To allow for the effective enforcement of traceability and labeling requirements, European legislation introduced the obligation for the applicant to provide, within the dossier, a reliable method for identification and quantification of the transformation event under authorization; the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) (*12*), established at the Joint Research Center of the European Commission and assisted by the European Network of GMO Laboratories (ENGL), has been given the task to evaluate the performance and reliability of the proposed method according to the guidelines described in the document "Definition of minimum performance requirements for analytical methods of GMO testing" (*6*).

If we consider that in the last few years the overall share of GM crops is constantly growing because of the emerging contribution of some developing countries, such as Argentina, Brazil, India, South Africa, and China (*13*), a significant increase in the number of GM food and feed circulating worldwide, including Europe, is expected. Consequently, European enforcement laboratories will be required to progressively expand their analytical capability for the detection, identification, and quantification of an increasing number of GMO events, with the consequence of making the analytical flow more and more complex, costly, and time-consuming. In this regard, improving practicability of GM food and feed official control in Europe is becoming an utmost priority.

At the time this paper was being written, 12 maize (*Zea mays*) GM events had already been authorized in the EU, while others are in the pipeline and a number of different methods for quantification of maize events have been submitted by the applicants and validated by the CRL. Because no constraints are set on the choice of the reference gene target, each company proposes its own method regardless of the presence of other already validated methods targeting the same taxon for similar quantitative purposes. The final result is sometimes an unbearable proliferation of methods.

From both a theoretical and technical point of view (except for very peculiar situations related to food matrix and amplicon length), there is no reason for changing the PCR reference system according to the GM event to be quantified. Therefore, the use of a unique taxon-specific PCR reference system for enforcement purposes is not only advisible for practical reasons but also sensible from a scientific perspective. The performance and reliability of several maize-specific methods have been tested thus far but largely focusing on the target gene more then the PCR system, with the objective of comparing intervarietal stability (*14*, *15*).

Table 2. Nanograms of Template DNA per Reaction and Corresponding Haploid Genome Copy Number for Each Concentration Level (from a to h)

concentration level	ng/reaction	copy number per reaction
a	100	36697
b	25	9174
c	6.25	2294
d	1.56	573
e	0.39	143
f	0.097	36
g	0.024	9
h	0.006	2

The aim of the present study was therefore to investigate the equivalence of five validated CRL methods for the maize reference gene, with the final goal of identifying a single maize-specific real-time PCR module to be coupled to several event-specific modules for the quantification of different transgenic maize events.

MATERIALS AND METHODS

Test Materials. Certified maize flours BT11, BT176, GA21, MON810, MON863, MON863 × MON810, and NK603 events (CRM-IRMM, Geel, Belgium) were used in this study. Varietal composition of maize powder is shown in **Table 1** according to each certification report.

DNA Extraction and Sample Preparation. DNA was extracted from 0.2 g of each maize flour using the CTAB method (*16*) and quantified spectrophotometrically (Biophotometer, Eppendorf, Hamburg, Germany). The DNA purity was checked by both calculating the ratio of the absorption values at 260/280 nm and carrying out a real-time PCR (monitor run) targeting zein encoding gene on two different amounts of each extracted DNA (200 and 50 ng).

Each extracted DNA (CRM1, CRM2, CRM3, CRM4, CRM4, CRM5, CRM6, and CRM7; see **Table 1**) was diluted up to 20 ng/μL and mixed with the others at equal volume, to obtain a pooled 20 ng/μL DNA solution (pool DNA).

Serial dilutions 1:4 of single CRM DNA and pool DNA were obtained as described in **Table 2**. The DNA haploid genome copy (HGC) number was calculated considering the amount of 1C maize DNA, corresponding to 2.725 pg (*17*).

Real-Time PCR Methods. Real-time PCR was carried out in optical 96-well reaction plate and run on the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Each reaction contained 5 μL of DNA sample.

In this study, the following protocols published by the CRL-GMFF (*12*) were used:

Method 1. Target gene *adh*1 (alcohol dehydrogenase): amplicon size, 70 bp; reaction volume, 50 μL (TaqMan Universal Master Mix 1×; primers, 150 nM; probe, 50 nM). Amplification profile: 50 °C, 120″; 95 °C, 600″; [95 °C, 15″; 60 °C, 60″] × 50 (data acquisition in the 60 °C step) [ADH 1 primer 1 (5′-CCA GCC TCA TGG CCA AAG-3′); ADH 1 primer 2 (5′-CCT TCT TGGCGG CTT ATC TG-3′); ADH 1 probe (5′-FAM-CTTAGGGGCAGACTCCCGTGTTCCCT-TAMRA-3′)] (*18*).

Method 2. Target gene *adh*1: amplicon size, 136 bp; reaction volume, 25 μL (TaqMan Universal Master Mix 1×; primers, 300 nM; probe, 200 nM). Amplification profile: 50 °C, 120″; 95 °C, 600″; [95 °C, 15″;

60 °C, 60″] × 50 (data acquisition in the 60 °C step) [ADH-F3 (5′-CGTCGTTTCCCATCTCTTCCTCC-3′); ADH-R4 (5′-CCACTCCGAGACCCTCAGTC-3′); ADH1-MDO (5′-FAM-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMRA-3′)] (*19*).

Method 3. Target gene *adh*1: amplicon size, 136 bp; reaction volume, 25 μL (TaqMan Universal Master Mix 1×; primers, 200 nM; probe, 200 nM). Amplification profile: 50 °C, 120″; 95 °C, 600″; [95 °C, 15″; 60 °C, 60″] × 50 (data acquisition in the 60 °C step) [KVM182 (5′-CGTCGTTTCCCATCTCTTCCTCCT-3′); KVM183 (5′-CCACTCCGAGACCCTCAGTC-3′); TM014 (5′-FAM-AATCAGGGCTCATTTTCTCGCTCCTCA-TAMRA-3′)] (*20*).

Method 4. Target gene *hmg*: amplicon size, 79 bp; reaction volume, 25 μL (TaqMan Buffer A 1×; $MgCl_2$, 4.5 mM; dATP, 200 μM; dGTP, 200 μM; dCTP, 200 μM; dUTP, 400 μM; AmpliTaq Gold DNA polymerase, 1 unit; primers, 300 nM; probe, 180 nM). Amplification profile: 95 °C, 600″; [95 °C, 15″; 60 °C, 60″] × 50 (data acquisition in the 60 °C step) [MaiJ-F2 (5′-TTGGACTAGAAATCTCGTGCTGA-3′); mhmg-rev (5′-GCTACATAGGGAGCCTTGTCCT-3′); Mhmg-probe: 5′-FAM-CAATCCACACAAACGCACGCGTA-TAMRA-3′) (*21*).

Method 5. Target gene *hmg*: amplicon, 79 bp; reaction volume, 25 μL (PCR Buffer II 1×; ROX reference Dye (Invitrogen, Carlsbad, CA), 0.7×; Tween 20, 0.01%; glycerol, 0.8%; $MgCl_2$, 5.5 mM; dATP, 200 μM; dGTP, 200 μM; dCTP, 200 μM; dUTP, 400 μM; AmpliTaq Gold DNA polymerase, 1 unit; primers, 400 nM; probe, 150 nM). Amplification profile: 95 °C, 600″; [95 °C, 15″; 60 °C, 60″] × 50 (data acquisition in the 60 °C step) [MaiJ-F2 (5′-TTGGACTAGAAATCTCGTGCTGA-3′); mhmg-rev (5′-GCTACATAGGGAGCCTTGTCCT-3′); Mhmg-probe: 5′-FAM-CAATCCACACAAACGCACGCGTA-TAMRA-3′)] (*22*).

Experimental Design. Single CRM DNA extracts were obtained and blended to form pool DNA as described above. Both pool DNA and single CRM DNA extracts were serially diluted, leading to 8 concentration levels from 36 697 to 2 haploid genome copies (HGC) per reaction (**Table 2**). Pool DNA dilutions were run with each of the five real-time PCR systems under evaluation, and each run was repeated 7 times, thus obtaining 5 (methods) × 7 (repetitions) = 35 regression curves from the pool DNA dilution set. Single CRM DNA dilution sets were also challenged with each of the 5 methods, thus leading to 7 (CRMs) × 5 (methods) = 35 regression curves (**Table 3**). All dilution levels were tested in five PCR replicates.

Real-Time PCR Data Analysis. Data were generated and analyzed by ABI Prism 7900 SDS software. Amplification plots were visualized in the logarithmic graph for manual setting of the fluorescent threshold value. This threshold was chosen in the middle of the linear phase of the PCR plots; the baseline was evaluated in the linear graph, by manual setting too.

After manual adjustment of these parameters, a regression curve for each DNA dilution set was obtained by plotting all of the Ct values, from each concentration level, against the log of the theoretical copy number of the corresponding concentration level. The regression curve Ct = a log(copy number) + b was used to calculate the experimental genome copy number corresponding to each specific Ct value. Ct values > 45 were rejected.

Relative repeatability standard deviation (RSD_r) was evaluated using 35 data points for each concentration level of pool DNA (seven repetitions of five replicates each).

Statistical Analysis. Each run was considered as a self-calibrating system; therefore, the experimental target copy number was calculated for all of the concentration levels, by deriving it from the regression curve itself. Hence, for each PCR system, from each concentration level, we obtained seven target copy number values from the pool DNA and one target copy number value from each of the seven single CRM DNA.

The values coming from the pool DNA were averaged, and the standard deviation was calculated. To exclude, at the 95% confidence level, that the pool DNA could provide biased results affecting the statistical evaluations, each calculated value, obtained from single CRM DNA, was verified to fall within the following range: $\bar{x}_{POOL,M,L} - (1.96 s_{POOL,M,L}) \leq x_{CRMn,M,L} \leq \bar{x}_{POOL,M,L} + (1.96 s_{POOL,M,L})$, where $\bar{x}_{POOL,M,L}$ is the average of the seven target copy number values obtained from pool DNA at a concentration level L using the PCR method M, $s_{POOL,M,L}$ is the standard deviation of the seven target copy number values obtained from pool DNA at concentration level L using the PCR method M, and $x_{CRMn,M,L}$ is the target copy number value obtained from CRM n DNA at concentration level L using the PCR method M.

Table 3. Experimental Design (5 × 7 = 35 Runs)

	Met 1	Met 2	Met 3	Met 4	Met 5
1	CRM1[a]/POOL[b]	CRM1/POOL	CRM1/POOL	CRM1/POOL	CRM1/POOL
2	CRM2/POOL	CRM2/POOL	CRM2/POOL	CRM2/POOL	CRM2/POOL
3	CRM3/POOL	CRM3/POOL	CRM3/POOL	CRM3/POOL	CRM3/POOL
4	CRM4/POOL	CRM4/POOL	CRM4/POOL	CRM4/POOL	CRM4/POOL
5	CRM5/POOL	CRM5/POOL	CRM5/POOL	CRM5/POOL	CRM5/POOL
6	CRM6/POOL	CRM6/POOL	CRM6/POOL	CRM6/POOL	CRM6/POOL
7	CRM7/POOL	CRM7/POOL	CRM7/POOL	CRM7/POOL	CRM7/POOL

[a] CRM DNA from 1 to 7 (see **Table 1**). [b] POOL DNA.

Grubbs test was used to identify possible outliers among calculated genome copy numbers for each concentration level.

For one-way analysis of variation (ANOVA), the significance level with a p value ≤0.05 was used as criterion to reject the null hypothesis "the five methods are not significantly different from each other". Before performing ANOVA, data were log-transformed.

Fisher's least significant difference (LSD) was used as a post-hoc test (after ANOVA) to find out which groups are significantly different from one another.

RESULTS AND DISCUSSION

Performance of Each Real-Time PCR Method for Maize Detection and Quantification. Unlike other studies published in the past few years on the comparison of maize reference PCR systems, focusing on the homogeneity of their response to a number of different cultivars, we decided to focus purely on method performance parameters. To compare PCR systems only, we deliberately decided to pool together the DNA extracted from those transgenic maize lines, which were available as CRM at the time this work was initiated. This approach tended to minimize the risk of introducing additional bias, because of possible biological differences from one maize variety to another in the target sequence or in the copy number of the target endogenous gene per haploid genome. This variability has already been investigated in previous studies (*14*, *23*) and should have been considered by applicants while evaluating specificity of the method that they intend to submit (*6*).

Furthermore, to have a statistically sound comparison of methods, we needed a large set of data. In this regard, the pool DNA allowed us to carry out a number of repetitions of the experimental procedure, where the only variable was the PCR method.

However, to exclude that the pool DNA could provide biased results, we decided to run also DNA dilutions of single CRMs with the five methods under investigation. Target copy number values of all of the dilutions of each CRM resulted to fall within the range $\bar{x}_{POOL,M,L} - (1.96 s_{POOL,M,L}) \leq x_{CRMn,M,L} \leq \bar{x}_{POOL,M,L} + (1.96 s_{POOL,M,L})$, thus indicating, at the 95% confidence level, no difference in the calculated copy numbers between single CRMs and pool DNA. Therefore, statistical analyses were carried out on pool DNA only.

The following parameters were investigated:

Regression Parameters. The slope and R^2 coefficient were determined for each regression curve. The slope is mathematically correlated to PCR efficiency according to the following equation: $E = 10^{-1/\text{slope}} - 1$, where E is the efficiency; 100% efficiency corresponds to a −3.32 slope value.

As shown in **Figure 1**, the mean slope value of method 4 turned out to be the closest to the −3.32 theoretical value, with a very low variability, whereas methods 1, 2, and 5 have a lower average slope value and a higher variability, and method 3 shows the smallest slope value, revealing a poor amplification efficiency, despite the very low variability.

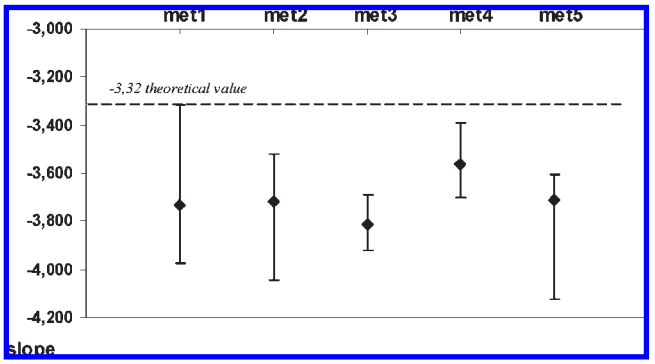

Figure 1. Slope range for each method. Bars represent maximum and minimum values, and squares report mean values. The dotted line corresponds to the theoretical value of −3.32.

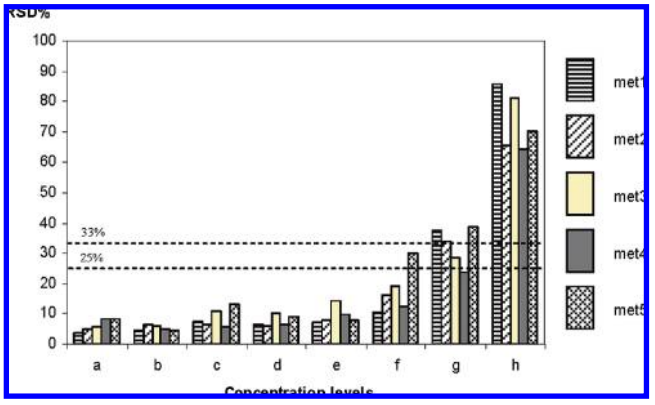

Figure 2. Relative standard deviation (RSD)% along the concentration range. Dotted lines correspond to 25 and 33% RSD. RSD% values of dilution g: 37% (method 1), 34% (method 2), 29% (method 3), 24% (method 4), and 39% (method 5). RSD% values of dilution f: 11% (method 1), 16% (method 2), 19% (method 3), 12% (method 4), and 30% (method 5). For dilutions, see **Table 2**.

The R^2 coefficient represents a measure of how well a linear regression line approximates the experimental data used to obtain it. The mean R^2 values were all between 0.995 and 0.999 (data not shown); thus, in terms of linearity, the five methods are all satisfactory and can be considered equivalent.

Relative repeatability standard deviation (RSD_r). RSD of the measured target copy number was calculated for a pool DNA dilution set under repeatability conditions (same operator, same instruments, on identical test items, and in a short interval of time). RSD_r gives information about method precision and is a useful tool for evaluating other validation parameters in the absence of collaborative trial data, as described later on. **Figure 2** displays the trend of RSD_r% along the concentration range for each method tested. As we expected, the lower the copy number, the less the precision of methods. Methods 2 and 4 exhibit a higher repeatability, although method 4 has a definitely better performance at low copy number values.

Limit of Detection (*LOD*) *and Limit of Quantification* (*LOQ*). The LOD can be defined as the minimum amount or concentration of the analyte in a test sample that can be detected reliably but not necessarily quantified, as demonstrated by a collaborative trial or other appropriate validation, whereas the LOQ is the lowest concentration or amount of the analyte in a test sample that can be quantitatively determined with an acceptable level of precision and accuracy, as demonstrated by a collaborative trial or other appropriate validation (*24*). Looking at the literature, for real-time PCR methods, we find numerous different approaches proposed and/or adopted for this purpose (*25*−*34*). Among all of these approaches, the most widely adopted consists of pointing out the lowest amount or concentration of the analyte, where it can be detected at least 95% of the time, ensuring ≤5% false negative results. **Table 4** shows the percentage of positive replicates for the dilutions from d (573 HGC) to h (2 HGC) for each method. According to this approach, the LOD of methods 1, 2, and 4 is 2 HGC and the LOD of method 3 and 5 is 9 HGC.

Table 4. Percentage of Positive Replicates for the Dilutions from d (573 HGC) to h (2 HGC) for Each Method (LOD Corresponding to 95% Positive Replicates)

dilution	Met 1 (%)	Met 2 (%)	Met 3 (%)	Met 4 (%)	Met 5 (%)
d (573 HGC)	100	100	100	100	100
e (143 HGC)	100	100	100	100	100
f (36 HGC)	100	100	100	100	98
g (9 HGC)	100	99	100	100	100
h (2 HGC)	95	95	81	95	92

An alternative strategy takes a cue from the ISO definition (*24*), whereby the LOD corresponds to the lowest level of analyte for which the relative standard deviation of reproducibility RSD_R should be 33% or less. In the lack of collaborative study data on maize endogenous reference systems, the RSD_R parameter is often not available. Considering that, in several validation data sets collected by the CRL-GMFF on maize event-specific real-time PCR methods, RSD_r and RSD_R show the same order of magnitude, we decided to apply the RSD_R 33% criterion to RSD_r to point out LOD of the five methods. According to this approach, the LOD of methods 3 and 4 is 9 HGC and the LOD of methods 1, 2, and 5 is 36 HGC (**Figure 2**).

With regard to LOQ, setting the cutoff of RSD_r at 25%, as indicated by the ENGL document (*6*), the LOQ of method 4 is 9 HGC, LOQ of methods 1, 2, and 3 is 36 HGC, and LOQ of method 5 is 143 HGC (**Figure 2**).

Comparison of the Calculated Genome Copy Number for Each Concentration Level. To further investigate the differences among methods, we compared the five PCR systems in terms of their capability of quantifying the maize genomic copy number by one-way ANOVA test. To set up the statistical test, we chose the concentration range from dilution a (36 697 genome copy number) to dilution e (143 genome copy number). This range is above the LOQ of all of the methods estimated as previously described and can be considered sufficient to meet the regulative requirements. **Figure 3** shows the mean copy number value and maximum and minimum values for each concentration level of each PCR system.

When ANOVA calculations are performed, certain assumptions have been made, in particular, homogeneity of variance. Considering that all of the measurements were made the same way by the same operator and the same instrument, we would expect homogeneity of variance (*35*). A second assumption was that the uncontrolled variations, such as temperature change or other factors that could produce a trend in the results over a period of time, were random. Moreover, to lead results to a normal distribution, data were log-transformed (*36*).

Before ANOVA was performed, outliers were identified by Grubbs test and no data were rejected.

Five ANOVA tests (one for each concentration level) were carried out on the calculated copy number. The tests reported that population means are not significantly different from each other, except at the lower concentration level (e, 143 HGC), where $p = 0.0235$.

ANOVA gives no information about which mean differs from the others. To point out the reason for a significant result, we used

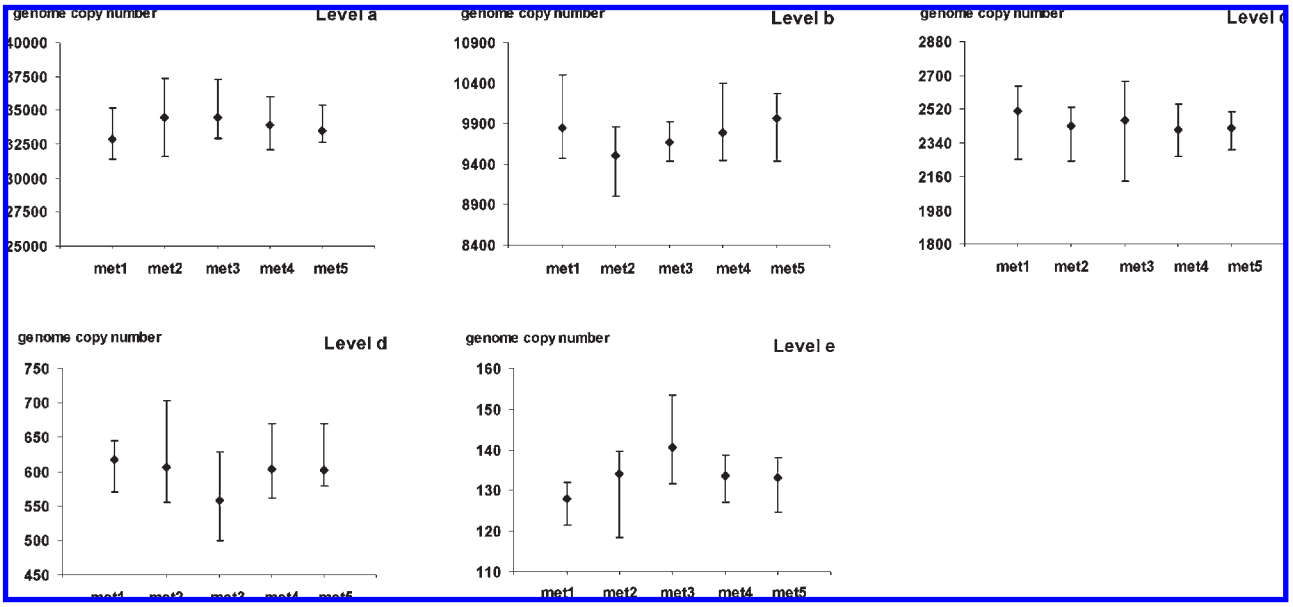

Figure 3. Mean copy number value for each method at different concentration levels. Level a, 36 697 haploid genome copy number (HGC); level b, 9174 (HGC); level c, 2294 (HGC); level d, 573 (HGC); level e, 143 (HGC). Bars represent the maximum and minimum values.

a post-hoc test, arranging the means in ascending order and comparing the difference between adjacent values with the Fisher's LSD. The results showed that method 3 differs from methods 1 and 5; no other significant differences were observed between the other groups.

On the basis of these results and statistical analysis, method 4, targeting the *hmg* gene, and method 2, targeting the *adh*1 gene, seem to be equivalent to each other and to the other methods in terms of quantification capabilities but method 4 shows the best regression curve parameters, the lowest LOD and LOQ, and a higher repeatability along the dilution range.

Hernández et al. (*14*) compared four real-time PCR systems targeting different maize sequences, namely, *adh*1, *hmg*, *ivr*1, and zein, and also took into consideration intervarietal variability of the target sequences. All of the methods proved to be very specific and accurate on a number of distantly related maize cultivars. Gene targets were also shown as being either single or low copy number genes.

More recently, Broothaerts et al. (*15*) have also tackled the issue of gene target stability, by reporting that the *adh*1 region exhibits a single nucleotide polymorphism (SNP839) that corresponds to a common allelic polymorphism in maize. As a result, the quantification of GM maize events could be positively or negatively biased, depending upon the *adh*1 genotype of the sample and calibrant.

Thus, considering both the variability observed within the *adh*1 sequence and the results of this work, we conclude that method 4, targeting the *hmg* region, looks to be the best candidate used as a "universal" maize reference gene system.

The PCR methods analyzed in this work were previously validated by the CRL-GMFF in combination with different event-specific methods (http://gmo-crl.jrc.ec.europa.eu/); this confirms their overall reliability and fitness for the purpose. By comparing these methods targeted at maize endogenous sequences, we identified a single *Z. mays*-specific method that, provided that the method modularity principle is endorsed, can be associated with different event-specific methods for the reliable detection of various maize events.

Method modularity is gaining a wider acceptance within the scientific community (*37*, *38*). Its implementation by an enforcement laboratory would allow for the validation of taxon-specific PCR methods as single modules and the adoption of these modules routinely in combination with an increasing number of event-specific modules for the official control of a growing number of GM events.

The possibility to identify a "joker" method to be used as a taxon-specific module for the quantitative detection of all GM events, as it is proposed in this work for *Z. mays*, looks promising. The impact on the optimization of the official controls, in terms of both harmonization and practicability, should not be neglected.

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