JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Validated Method for Quantification of Gentically Modified Organisms in Samples of Maize Flour

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Sensitive and accurate testing for trace amounts of biotechnology-derived DNA from plant material is the prerequisite for detection of 1% or 0.5% genetically modified ingredients in food products or raw materials thereof. Compared to ELISA detection of expressed proteins, real-time PCR (RT-PCR) amplification has easier sample preparation and detection limits are lower. Of the different methods of DNA preparation CTAB method with high flexibility in starting material and generation of sufficient DNA with relevant quality was chosen. Previous RT-PCR data generated with the SYBR green detection method showed that the method is highly sensitive to sample matrices and genomic DNA content influencing the interpretation of results. Therefore, this paper describes a real-time DNA quantification based on the TaqMan probe method, indicating high accuracy and sensitivity with detection limits of lower than 18 copies per sample applicable and comparable to highly purified plasmid standards as well as complex matrices of genomic DNA samples. The results were evaluated with ValiData for homology of variance, linearity, accuracy of the standard curve, and standard deviation.

KEYWORDS: 35S promoter; GMO; maize; TaqMan; ValiData

INTRODUCTION

Maize (*Zea mays* L.) is grown primarily for its kernel, which is largely refined into products used in a wide range of food, medical, and industrial goods. Only a small amount of whole maize kernel is consumed by humans. Maize oil is extracted from the germ of the maize kernel, and maize is also used in the manufacture of starch. Refined maize products, sweeteners, starch, and oil are abundant in processed foods such as breakfast cereals, dairy goods, and chewing gum.

In the United States and Canada maize is typically used as animal feed, with roughly 80% of the crop fed to livestock. Animals that feed on maize include cattle, pigs, poultry, sheep, goats, fish, and companion animals.

The European Union (EU) dictates the scientific evaluation for the permission of genetically modified organisms (GMO) in human food as well as feeding stuff to generate a concise and transparent system. Since 2004 all food comprising GMO or prepared from GMO has to be indicated independently of the traceability of the GMO in the final product. This strategy is encoded in EU Regulation 1830/2003 regulating the labeling and retracing of the whole production process. In the case of genetically modified material that is not approved in the EU, despite a positive scientific estimation, the threshold value to be inserted into food is 0.5%. This relative percentile quantification requires the absolute quantification of the distinct DNA species in the sample and is usually accomplished by spectrophotometrical DNA detection also used in our laboratory. An alternative is introduced by Hernandez et al. (*1*), who reported different endogenous genes of the species as reference point for DNA quantification in an unknown variety (*2*).

The principal traits in most of the GM maize breeds are herbicide and insect tolerance (*3*) under control of Cauliflower mosaic virus 35S promoter (CaMV 35S) (*4*). In this paper we describe a method for the quantification of the 35S promoter in maize flour, based on a plasmid standard curve with a sensitivity of 18 copies per sample. The advantage of this validated method is the independence from the sample matrix due to TaqMan probes and the easy handling of standards. In our example we used a 162 bp amplicon of the 35S promoter for quantification and confirmed the sequence homology for different GM maize species to guarantee equal PCR efficiencies necessary for correct quantification. The real-time (RT) PCR amplification is described by an exponential function that is reduced to a first-order function by taking the logarithm of the starting concentration. Therefore, the results have to be validated carefully; otherwise, the variation of final results is too high. The interpretation of the raw data and conversion to the gene copy number of starting material are discussed in detail.

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10.1021/jf052257s CCC: $33.50 © 2006 American Chemical Society
Published on Web 01/17/2006

MATERIALS AND METHODS

BCR Standards. Cornmeal samples of maize BT11 (5% GMO), NK603 (5% GMO), and MON810 (5% GMO) reference standards were received from the Bureau Communautaire de Rèferènce (BCR; developed at the Institute for Reference Materials and Measurements, Geel, Belgium, commercialized by Fluka), purified by the CTAB method (*5*) and diluted to 100 ng/μL.

Genomic DNA sample, AM32, was provided by Agrana Maisstärkefabrik (Aschach, Austria) in a concentration of 100 ng/μL. In brief, 2 g of homogenized cornmeal was diluted in 15 mL of CTAB extraction buffer (20 g/L hexadecyltrimethylammonium bromide, 81.8 g/L NaCl, 5.8 g/L EDTA, pH 8.0), incubated for 60 min at 65 °C, and centrifuged for 10 min at 12000*g*. Five milliliters of the upper layer was extracted with 4 mL of chloroform. After 30 s of mixing, the mixture was centrifuged for 15 min at 12000*g*. Four milliliters of supernatant was transferred into a new tube, and 2 volumes of CTAB precipitation solution (5 g/L CTAB, 2.34 g/L NaCl) was added. The mixture was incubated for 60 min at room temperature and then centrifuged for 10 min at 12000*g*. The supernatant was removed, and the precipitate was dissolved in 1.2 M NaCl and chloroform extracted; 2.5 mL of the upper layer (aqueous phase) was transferred to a new tube, and 0.7 volume of 2-propanol was added. After centrifugation, the resulting DNA pellet was washed with ice-cold 70% ethanol and centrifuged. The ethanol was discarded, and residual ethanol was removed in a vacuum exsiccator; the DNA pellet was resuspended in 100−500 μL of TE buffer.

Plasmid standards were generated by amplification of 35S promoter with 35S-7245 sense 5′-gaattcccgacagtggtcccaaagatgg-3′ and 35S-7406 antisense 5′-gcggccgcatatagaggaagggtcttgc-3′ primers and cloned into the *Eco*RI/*Not*I opened pBluescript (Stratagene, La Jolla, CA) vector. Inserts of standard genomic DNAs (NK603, BT11, and MON810) were sequenced using the standard chain termination method (*6*) and aligned to the 35S promoter of cauliflower mosaic virus genome (Genbank accession no. V00140, J02046). Plasmids containing *Escherichia coli* Top 10 were propagated in ampicillin containing LB medium, and plasmids were purified via Qiagen Tip 100 (Qiagen, Norway).

Establishment of Plasmid Standard Stock Solution. Plasmid DNA was quantified spectrophotometrically at 260 nm, diluted to a concentration of 10E4 copies/μL (33.9×10^{-6} ng/μL), and stored in aliquots at −20 °C.

RT-PCR with TaqMan Probes. RT-PCR assays were carried out in an iCycler (Bio-Rad Laboratories, Hercules, CA), using the TaqMan system in a final volume of 25 μL; all primers and probes were synthesized at Invitrogen. The reaction mix included 12.5 μL of iQsupermix (Bio-Rad) 0.5 μM forward primer, 35S-7248 sense 5′-ACAGTGGTCCCAAAGATGGA-3′, 0.5 μM antisense primer, 35S-7396 antisense, 5′-AGGGTCTTGCGAAGGATAGT-3′, and 0.5 μM fluorogenic probe, 35S-7269 sense-probe, 5′-CCCCACCCACGAG-GAGCATCG-3′, labeled with 6-carboxyfluorescein (FAM) at the 5′-end and with the fluorescent quencher dye 6-carboxytetramethyl-rhodamin (TAMRA) at the 3′-end. Primers used in this assay were designed using Primer 3 software (*7*). Conditions for amplification were 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Each sample including all controls and points from the standard curve was quantified in triplicates.

Validation of Results. Statistical control of the linear model was performed according to International Conference on Harmonisation (ICH) guidelines (ICH Harmonised tripartite Guidelines, 1996 ICH steering committee, "Validation of analytical procedures: methodology Q2B") with the ValiData software (*8*).

Different parameters have been calculated to validate this method; those specifying the standard curve and the sample detection are summarized here:

1. The linear range of the calibration standard curve equation was tested according to ValiData.

2. The limit of detection (LOD) as well as the limit of quantification (LOQ) was estimated by ValiData according to the calibration curve method as a multiple of the standard deviation.

3. The variance was detected for highest and lowest concentration of the curve and judged at 95 and 99% significance levels in ValiData.

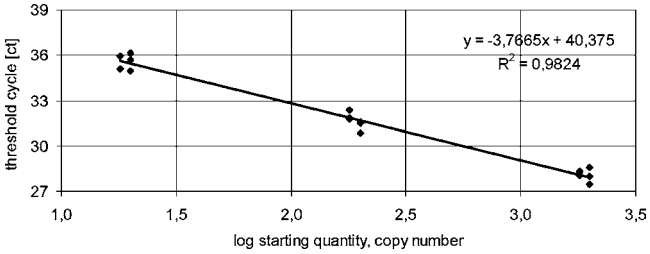

Figure 1. Amplification plot of real-time calibration curve including plasmid standards and GMO standards. The correlation between cycle number (ct) and initial copy number of 35S promoter is described by the interception of 40.4, the correlation coefficient ($R^2 = 0.98$), and slope of the curve: −3.77. Dots indicating plasmid standards are described by log starting concentrations of 3.3, 2.3, and 1.3; those of the 5% GMO MON 810 have log starting quantities of 3.26, 2.26, and 1.26.

4. The relative standard deviation (% CV) and the recovery (% *r*) are defined as

$$\%\ \mathrm{CV} = \frac{100\sigma}{m}$$

$$\%\ r = \frac{100m}{\text{predicted} - \text{copies}}$$

where m is the mean and σ is the standard deviation.

RESULTS

Sequence Homology of GMO Standards. Plasmids containing the amplified 162 bp fragment of the 35S promoter were sequenced, and 100% homology was shown for all inserts amplified from NK603, BT11, and MON810 according to the cauliflower mosaic virus genome (*9*, *10*). Additionally, positively tested DNA samples provided by Agrana Maisstärkefabrik were also shown to have identical 35S promoter fragments.

Verification of Standards. The plasmid standard calibration curve was verified by comparison of diluted plasmids with 5% GMO MON810 genomic DNA samples. Plasmid standards containing 2000, 200, and 20 copies and MON810 5% GMO genomic DNA samples (1800, 180, and 18 copies) were used as template in the same RT-PCR experiment, and the plasmid standards as well as MON810 standards comprise the standard curve. **Figure 1** shows the correlation coefficient and the slope of the curve as well as the interception when using all six different standard sample concentrations analyzed in triplicate. The correlation coefficient was estimated to be 0.98, giving clear evidence for the accuracy of the independently prepared samples and the method. The amplification efficiency is demonstrated by the slope of the curve, indicating 87% efficiency resulting from a slope of −3.7. Validation of these results with ValiData confirmed the linearity of the calibration curve. Additionally, the same conclusion gave the check of variances of highest and lowest values at 95 and 99% levels of significance. The LOD and the LOQ were indicated by 2.3 and 3.9 copies, respectively.

The residual analysis of all calibration points is shown in **Figure 2**, showing no deviation of the Gaussian distribution and no trend across the calibration curve. **Figure 3** describes the calibration interval and the prediction interval based on the data of the standard curve.

Quantification of Sample AM32. The gene copy number of 35S promoter in sample AM32 was determined in three different experiments. **Table 1** summarizes the results obtained using the plasmid calibration curve. The average result of these analyses gave 25 copies of 35S promoter in 200 ng of genomic DNA, resulting in 0.07% GM maize in sample AM32.

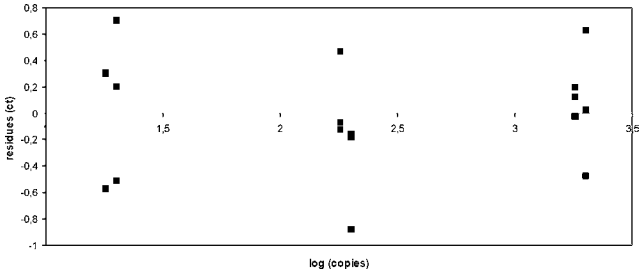

Figure 2. Analysis of residues of the calibration curve.

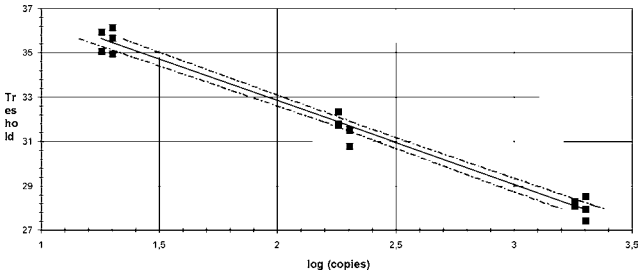

Figure 3. Calibration curve with confidential interval and interval of prognosis.

Table 1. Quantification of 35S Promoter Gene Copy Numbers in 200 ng of AM32 Genomic DNA in Three Independent Experiments in Triplicate and Quadruplicate, Respectively

	single values (copies)			
parallel analyses	1	2	3	4
expt 1	24	12	34	12
expt 2	33	37	41	19
expt 3	25	12	25	
mean		25		
SD		10.44		

Assessment of Matrix. In this experiment plasmid standards with a concentration of 2000, 200, or 20 copies per 2 μL of template were used for quantification. Two artificial samples were generated by mixing plasmid standards and genomic corn DNA samples. Artificial sample 1 (AS1) was generated by mixing 18 μL of AM32 (with a content of <0.5% GMO) with 2 μL of the 1000 copies/μL plasmid standard; AS2 consisted of 5 μL of BT11 DNA (5% GMO BT11 in corn meal mixture) plus 5 μL of 100 copies/μL plasmid standard; 2 μL of DNA template was used in each experiment for artificial samples, pure GMO samples, and plasmid standards. In theory, 2 μL of the BT11 sample contains 360 copies of the 35S promoter amplicon in the dilution 1:5.

Table 2 explains the samples, the raw data indicated by threshold cycles, and predicted copy number of 5% GMO BT11 standard and artificial samples as well as the results generated by the plasmid calibration curve analyzed in triplicates (BT11, AS1, and AS2) and mean values thereof. The concentration of AS1 is not defined because we do not know the distinct concentration of this real sample. We have determined the portion of GMO in sample AM32 with 25 copies per 200 ng of DNA, clearly indicated to be lower than the 0.5% GMO limit. Calculation with this analyzed value gives a prediction of 222.5 copies for AS1. In the case of the sample 5% GMO BT11 the theoretical value is 360 copies in this preparation, and the generated results were dispersed between 249 and 430 copies. However, the mean value indicated good accordance with the predicted value. Similar dispersion was evident for the samples AS1 ranging from 149 to 285 copies in this approach and AS2 with a dispersion of 799−1300. Despite this dispersion, mean values of independent analysis of AS1, AS2, and 5% GMO BT11 gave good accordance with the predicted gene copy numbers. All data generated in this experiment were validated for their statistical relevance.

Table 2. Statistical Examination of the RT-PCR Quantification in Different Matrices[a]

	5% GMO BT11 1:5				AS1			AS2	
Statistical Analysis of ct Values									
ct[b]	30.93	31.77	31.05	32.46	31.56	32.64	29.22	29.74	29.97
RSD		1.45			1.80			1.30	
Statistical Analysis of the Log Gene Copy Number Reflecting the Raw Data									
predicted log copies		2.56			2.35			3.00	
copies/sample[c]	2.63	2.40	2.60	2.20	2.45	2.15	3.11	2.97	2.90
mean		2.54			2.27			2.99	
RSD		5.04			7.21			3.61	
recovery (%)		99.47			96.64			99.83	
Statistical Analysis of Final Copy Number									
predicted copies		360			222.5			1000	
copies/sample[c]	430	249	397	159	285	141	1300	929	799
mean		359			195			1009	
RSD		26.88			40.24			25.76	
recovery (%)		99.63			87.64			100.93	

[a] Samples used were 5% GMO BT11; AS1, consisting of 5% GMO BT11 and plasmid standard; and AS2, consisting of sample AM32 and plasmid standard. Statistical parameters [mean value, relative standard deviation (RSD), and recovery] were calculated for the results demonstrated as ct values, the logarithm of gene copy number, and copies per sample. [b] Threshold cycles. [c] Single values from analyses in triplicate.

Validation of Data. The RT-PCR interpretation of initial gene copy number in genomic DNA samples occurs via a linear standard curve generated by logarithms of starting concentration of the target gene correlated to the threshold cycle, indicated by the detection of a distinct fluorogenic signal. This standard curve was verified by comparison of first- to second-order curves, resulting in confirmation of the linear range of the standard curve.

The LOD and LOQ were defined and allow a quantification of GMO lower than 0.5% GMO in 200 ng of genomic DNA indicated by a threshold of 182 copies. However, the approved LOQ allows the detection of <0.1% GMO in 200 ng of maize genomic DNA.

The matrix exploration experiment was validated for accuracy and recovery, yielding 87.6−100.9% recovery of predicted values, indicating a very efficient quantification of samples (**Table 2**).

The statistical spread is demonstrated best by the relative standard deviation (RSD) and is calculated to be <1.8% for ct values in three independent measurements with no indication for abnormal statistical variation (**Table 2**). The read-out of these ct values is the "log starting quantity" (compare **Figure 1**), and the RSD was calculated to be 3.61−7.21% in our quantification model, a rather good result for such a complex method. However, the PCR is an exponential method, and inversion of the logarithm of starting quantity leads to a higher spread of the final results also shown in **Table 2**. In case of trace amounts of GMO in maize flour it is not recommended to state the absolute gene copy number, but rather to define the threshold with <0.5% GMO in the sample (compare **Table 1**).

Taken together this validated quantification meets all demands for the absolute quantification of GMO in maize flour with amounts >0.5% of the transgene.

DISCUSSION

In this paper we describe a method for the detection of GMO in real maize samples according to EU Regulation 1830/2003 based on a 0.5% limit. Different strategies to detect transgenic DNA have been described (e.g., amplification of fragments of the 35S promoter, the NOS ending, and the junction of coding sequence and regulatory sequence). However, any of these methods is uncertain, and the quantification depends on the determination of transgene and the total copy number of DNA (*11*).

The relevant issues in real-time quantification of the GMOs are the choice of adequate primers, probes, and evaluation model, the appropriate standard material, and the DNA isolation method. The DNA extraction method should be cost- and time-effective, especially in the case of high amounts of samples. Previous examinations of different commercially available kits based on silica gel, magnetic beads, and precipitation were compared to lysis and precipitation with CTAB, showing that the conventional CTAB purification gave highest yield with sufficient PCR amplification protocols (*12*). The standard curves are predominantly generated from BCR GMOs in concentrations between 5 and 0.1% GMO in the corn meal (*13*, *14*). The use of genomic DNA for the calibration curve is essential for the SYBR green method, where the fluorophor intercalates with the entire double-stranded DNA generated during amplification and unspecific amplicons in trace amounts may falsify the results. Therefore, the SYBR green method is sensitive to matrix effects, and it is not possible to dilute one distinct concentration of the BCR standard for the generation of a standard curve (data not shown), but rather a whole series of standards has to be prepared, controlled for purity, and quantified. Besides the real-time experiments we have also sequenced the 162 bp long amplicon of CaMV 35S promoter and found 100% homology for BT11, NK603, and MON810, giving evidence for the quantification of real samples with these primers. Additionally, we compared the amplicons of the invertase exon from three of our breedings with the Genbank entry (GI 1122438). In the cases of NK603 and MON 810 we found some sequence divergence leading to different PCR efficiencies that result in problems with the interpretation of quantification.

Although the quantification of a housekeeping gene is recommended by some authors for exact quantification of the target, there are some drawbacks, mainly because of additional imprecision in the RT quantification, generated either by the dilution of the DNA or by differences in the fluorescence emission in the reports (*1*). In our case we prefer the conventional spectrophotometrical DNA quantification and use the amplification of invertase as positive control to approve the DNA quality.

The plasmid standards enable more flexibility and accuracy in preparation but still show the same amplification efficiency as complex samples in the system. The RSD of ct values is constant and reproducible along the whole calibration interval, and the genomic DNA samples from maize flour did not reveal significant differences after a couple of freeze−thawing cycles. Therefore, we conclude that the CTAB purification method is well suited for generating sufficient amounts of DNA with adequate quality. This validated method combines the potential of the TaqMan probe method with the easy handling of a plasmid calibration curve.

ACKNOWLEDGMENT

We thank Christine Lattenmayer and Sonja Preis for perfect technical support.

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Received for review September 13, 2005. Revised manuscript received November 2, 2005. Accepted November 7, 2005. This work was kindly sponsored by Agrana Maisstärkefabrik.

JF052257S