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Distortion of Genetically Modified Organism Quantification in Processed Foods: Influence of Particle Size Compositions and Heat-Induced DNA Degradation

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Milling fractions from conventional and transgenic corn were prepared at laboratory scale and used to study the influence of sample composition and heat-induced DNA degradation on the relative quantification of genetically modified organisms (GMO) in food products. Particle size distributions of the obtained fractions (coarse grits, regular grits, meal, and flour) were characterized using a laser diffraction system. The application of two DNA isolation protocols revealed a strong correlation between the degree of comminution of the milling fractions and the DNA yield in the extracts. Mixtures of milling fractions from conventional and transgenic material (1%) were prepared and analyzed via real-time polymerase chain reaction. Accurate quantification of the adjusted GMO content was only possible in mixtures containing conventional and transgenic material in the form of analogous milling fractions, whereas mixtures of fractions exhibiting different particle size distributions delivered significantly over- and underestimated GMO contents depending on their compositions. The process of heat-induced nucleic acid degradation was followed by applying two established quantitative assays showing differences between the lengths of the recombinant and reference target sequences (A, $\Delta l_A = -25$ bp; B, $\Delta l_B = +16$ bp; values related to the amplicon length of the reference gene). Data obtained by the application of method A resulted in underestimated recoveries of GMO contents in the samples of heat-treated products, reflecting the favored degradation of the longer target sequence used for the detection of the transgene. In contrast, data yielded by the application of method B resulted in increasingly overestimated recoveries of GMO contents. The results show how commonly used food technological processes may lead to distortions in the results of quantitative GMO analyses.

KEYWORDS: GMO; quantification; PCR; particle size; degradation

INTRODUCTION

The use of genetically modified organisms (GMOs) and GMO-derived products in the food chain is subject to regulatory provisions in a number of countries (*1*). In the European Community, a uniform traceability system defining provisions for the documentation of the flow of GMO-derived commodities has been established (*2*, *3*). Data gained by this system build the basis for labeling, which is no longer triggered by positive testing of products for the presence of GMO materials. By this means, even highly processed GMO-derived products are covered by the new legislation, irrespective of the detectability of recombinant DNA or proteins. Nevertheless, development and validation of quantitative methods for GMO analysis in foods remain essential, because thresholds have been set, e.g., 0.9% of the food ingredients considered individually (*2*), acknowledging that adventitious contaminations of products with GMO material cannot be excluded.

The analysis of processed foods has been repeatedly shown to entail a number of complications, which negatively affect the performance of GMO detection methods. Complications emerge from food manufacturing steps, which may involve complex enzyme-catalyzed or chemical reactions as well as rather simple mechanical procedures, e.g., milling. The first part of this paper deals with the influence of the compositions of samples containing different corn milling fractions on the relative quantification of GMO contents. Mixtures of milling fractions with different particle size distributions are usually used in industrial applications, for instance, in bakery and extrusion products, to regulate important functional and sensory characteristics (*4*, *5*). Commonly used milling fractions [coarse grits, regular grits, cornmeal, and flour (*4*)] were therefore manufactured in laboratory scale. Samples prepared by different combinations of coarse grits and flour from conventional or transgenic corn (all adjusted to a GMO content of 1%) were

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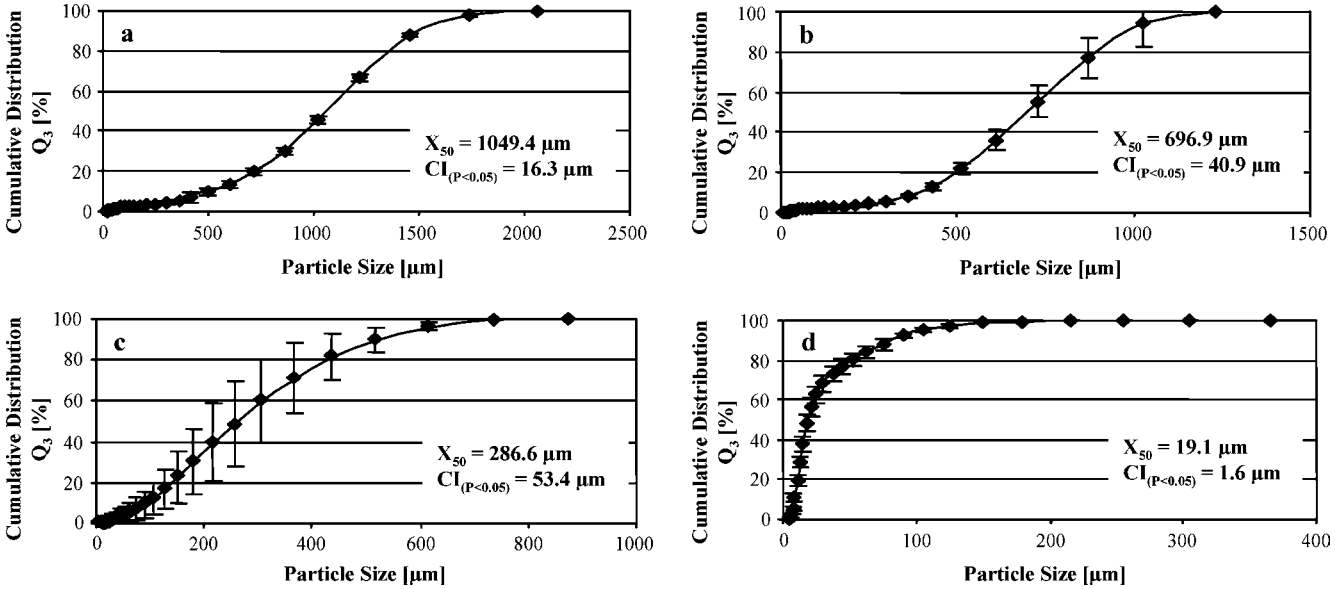

Figure 1. Cumulative particle size distributions of the corn milling fractions prepared at laboratory scale. (**a**) Coarse grits (sieve, 1 mm), (**b**) regular grits (sieve, 0.8 mm), (**c**) meal (sieve, 0.4 mm), and (**d**) flour (sieve, 0.1 mm). For optimal visualization, the scales of the *x*-axes are adjusted to the expected particle size ranges. $CI_{(P<0.05)}$: confidence interval at 95% probability.

subsequently quantified using a commercially available real-time polymerase chain reaction (PCR) quantification kit.

Food manufacturing also involves a number of processing steps, which take place under harsh physicochemical conditions thus affecting the integrity of genomic DNA in tissues and/or matrices of individual food ingredients. As shown in previous studies, degradation of DNA is primarily linked to processes carried out at low pH values and increases dramatically if these are performed in combination with thermal stress (*6*−*9*). Additionally, extensive DNA fragmentation has been observed as result of enzymatic hydrolysis (*10*−*12*) [e.g., in fermentation processes (*13*)] and to a certain extent during grinding and milling processes owing to shear forces and further mechanical stress (*14*).

Effects of DNA degradation on the results of PCR-based detection systems have been investigated by following diverse manufacturing practices. It was shown that the lengths of the selected target sequences are crucial parameters for the detectability of DNA (*7*, *13*, *15*−*18*). Qualitative assays demonstrated that longer target sequences (>300 bp) become increasingly susceptible to degradation under stress conditions, thus leading to false negative results when analyzing samples of processed foods or feeds. Subsequent quantitative competitive PCR approaches confirmed these conclusions, revealing significant decreases in the recoveries of target sequences in the course of processing (*12*, *19*). Consequently, for quantitative approaches, the importance of using plant specific reference genes that allow the normalization of quantification results was emphasized as a requirement for the determination of ingredient-related GMO contents (*20*).

On the basis of these observations, degradation of DNA was estimated to have adverse effects on the performance of quantitative assays, affecting limits of detection as well as limits of quantification (*21*). The improved stability of shorter DNA sequences toward stress conditions resulted in a commonly acknowledged approach to aim at target sequences ≤200 bp in qualitative and quantitative assays. These assays were generally considered as appropriate for the analysis of processed products, as it was assumed that recombinant and reference target sequences possessing approximately the same length will be equally degraded in the course of processing (*22*).

Table 1. Compositions of Sample Mixtures (1% w/w Bt176 Corn)

	Bt 176 corn		conventional corn	
sample	coarse grits (mg)	flour (mg)	coarse grits (mg)	flour (mg)
mix 1	3		297	
mix 2		3		297
mix 3		3	297	
mix 4	3			297

Table 2. Influence of Particle Sizes on DNA Extraction Yields

		DNA extracts (ng/µL)[a]	
milling fraction	median particle size x_{50} (µm) ± $CI_{(P<0.05)}$	CTAB extraction	Wizard extraction
coarse grits	1049 ± 16	196	200
regular grits	697 ± 41	173	236
meal	287 ± 53	320	347
flour	19 ± 2	527	359

[a] Starting from 300 mg sample; means of duplicate measurements.

Currently used methods for the relative quantification of recombinant DNA are based on the widely established real-time PCR technology. Development and validation are generally accomplished by analyzing certified reference materials (Institute of Reference Materials and Measurements, Belgium), which represent mixtures of flours containing defined proportions of GMO-derived material. Following accuracy testing, quantitative assays have been directly applied to the analysis of processed foods. However, experiments demonstrating that neither food composition nor processing influence the trueness of relative GMO quantification have not been performed (*23*−*27*), thus neglecting a principal aim of the implemented regulations, i.e., the surveillance of GMO contents in composed and processed foods. Some studies focused on the analysis of processed samples with known GMO proportions but did not determine the GMO proportions of the unprocessed counterparts (*28*−*30*). This left the uncertainty whether the found discrepancies in quantification results were induced as a consequence of DNA degradation or if these are to be seen as inherent bias of the quantification system. Results of an international ring trial regarding the validation of a method for the quantification of transgenic maize (line Bt176) allowed an accurate quantification

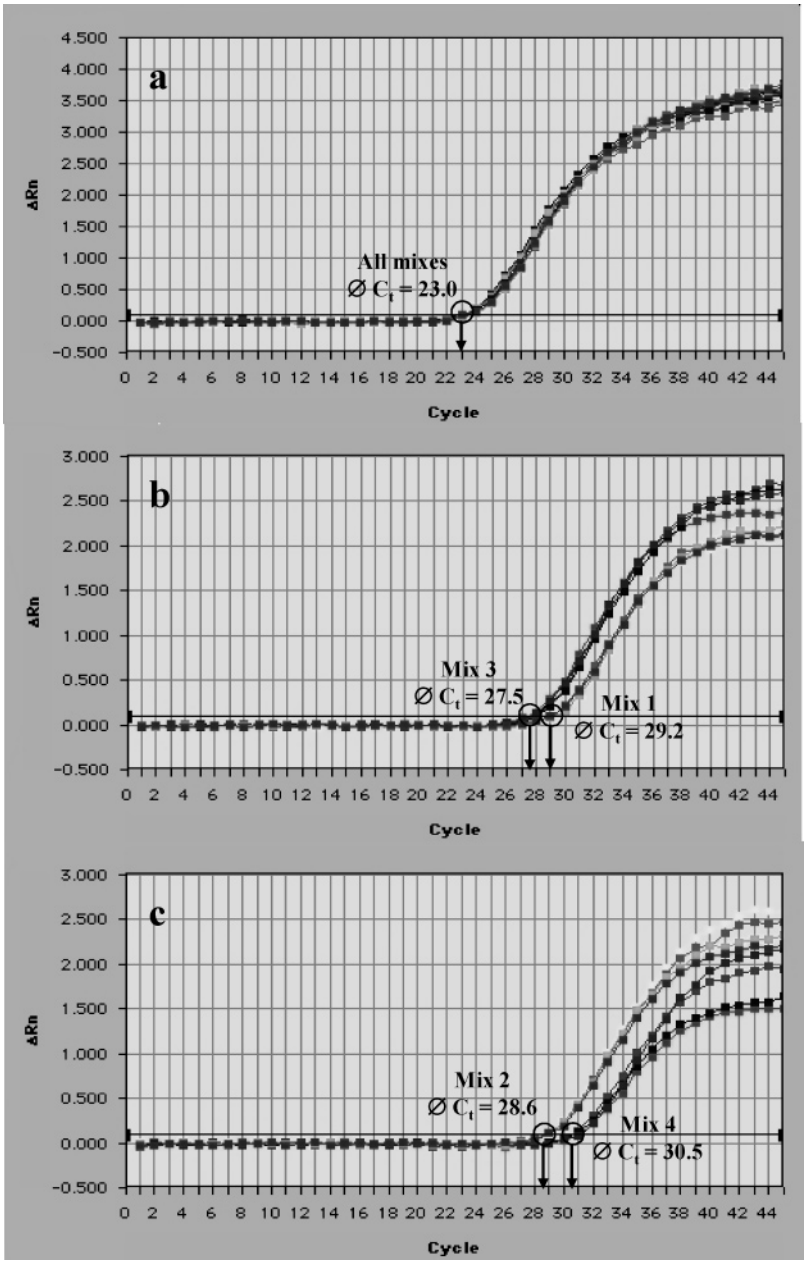

Figure 2. Amplification plots of the extracts from mixes 1–4 (**Table 1**) after solid phase DNA preparation (Wizard method). (**a**) *HMGa* reference gene, all mixes; (**b**) *CrylA(b)* gene, mixes 1 and 3; and (**c**) *CrylA(b)* gene, mixes 2 and 4. ΔRn = normalized reporter fluorescence; Ø C_t = average threshold cycles.

of GMO contents in unprocessed reference materials but showed a significant underestimation of GMO contents in heat-sterilized samples (*31*, *32*). The fact that the transgenic specific target sequence (129 bp) was longer than that used for the detection of the reference gene (79 bp) permitted the assumption that distortions in the results of relative quantification could result from an unequal sensitivity of target sequences toward processing parameters.

The objective of the following studies was to elucidate the influence of (i) particle size distributions in composed foods and (ii) nucleic acid degradation in processed products on the relative quantification of recombinant DNA via real-time PCR. Milling fractions produced from genetically modified Bt176 corn were thermally treated and analyzed using a commercially available quantification kit (method A) and an additional quantification method (*25*) (method B). Experiments were designed to assess whether minor differences in the lengths of the targeted sequences of recombinant and reference genes occurring in these methods ($\Delta l_A = -25$ and $\Delta l_B = +16$ bp, related to the respective amplicon length of the reference gene) may cause different stability toward thermal stress and consequently distortions in the results of relative quantification. Different milling fractions (coarse grits and flour) were analyzed to obtain information on the influence of the sample matrix on the rates of DNA degradation.

MATERIALS AND METHODS

Material. Seeds of genetically modified, insect resistant Bt corn (Event 176, breeding line: Navares) and of the corresponding conventional corn (Antares) were provided by the Bavarian Institute for Animal Production (Bayerische Landesanstalt für Tierzucht, Grub, Germany).

Milling Products. Corn kernels (100 g batches) were ground for 30 s in a laboratory blender. The resulting material was fractionated using 1, 0.8, 0.4, and 0.1 mm standard sieves (DIN 4188, ISO 565

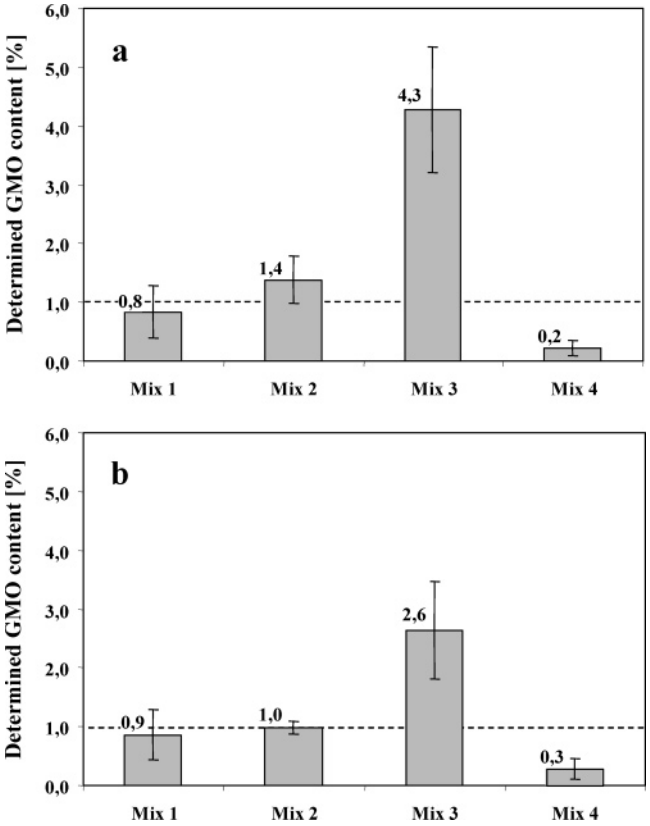

Figure 3. Quantification results of flour/grits mixes 1–4 (**Table 1**) after DNA extraction with (**a**) CTAB method and (**b**) Wizard method. Means ± $CI_{(P<0.05)}$. The dotted lines indicate the adjusted GMO content of 1%.

T1). The particle size distributions of the obtained fractions were measured using a laser diffraction system (Helos, Sympatec GmbH, Clausthal/Zellerfeld, Germany). Two dry dispersion techniques were applied; a fall-shaft gravity disperser (GRADIS) was used for the 1, 0.8, and 0.4 mm fractions, and a shear-force, jet nozzle disperser (RODOS) was used to measure the particle size distribution of the 0.1 mm fraction. Triplicate measurements were performed for each fraction.

Mixtures of Milling Fractions. The milling fractions were dried for 48 h at 55 °C to equalize moisture contents. Mixtures between milling products from conventional and transgenic material (corn coarse grits and flour) were prepared directly in 2 mL reaction vessels. As described in **Table 1**, the proportion of 1% Bt 176 corn was obtained by using four different mixing regimes. Four sorts of mixtures were generated by adding 3 mg of grits or flour from transgenic maize to 297 mg of grits or flour from conventional maize. Four samples of each mixture sort were analyzed.

Heat-Treated Samples. Two grams of coarse grits and flour from maize Bt176, respectively, was filled in three reaction vessels (50 mL) and mixed with 4 mL of H_2O. One sample was used as the control and remained untreated, while the other two samples were subjected to heat treatment (60 and 120 min, respectively) in boiling water. Samples were subsequently dried overnight at 45 °C and ground separately in a mortar to obtain a homogeneous powder. Each sample (300 mg) was distributed in 2 mL reaction vessels for DNA extraction.

DNA Extraction. Two methodologies were applied to extract DNA from the sample mixtures: (i) The Wizard extraction (*33*) was performed by adding 860 μL of extraction buffer [10 mM Tris-HCL, 150 mM NaCl, 2 mM EDTA, and 1% (w/w) sodium dodecyl sulfate (SDS), pH 8.0], 100 μL of 5 M guanidine chloride, and 40 μL proteinase K (20 mg/mL) to each sample (300 mg) and incubating the mixture at 60 °C for 3 h. After incubation, mixtures were centrifuged at 12000*g* for 10 min. The supernatant (500 μL) was transferred into another tube, mixed with 5 μL of RNase A (10 mg/mL), and incubated at 60 °C for 5 min. The extracted DNA was purified according to the isolation protocol (*34*), using Wizard DNA binding resin (Promega, Madison, WI), and finally eluted in 100 μL of elution buffer (10 mM Tris HCL, pH 9).

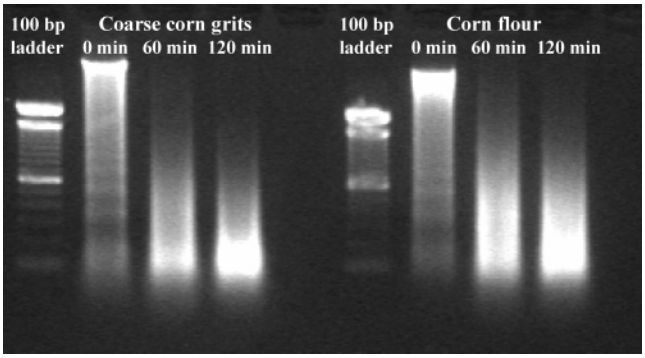

Figure 4. Degradation of DNA in coarse corn grits and corn flour samples induced by thermal treatment for 60 and 120 min at 100 °C.

(ii) The CTAB extraction (*35*) was performed by adding 1000 μL of extraction buffer [cetyltrimethylammonium bromide (CTAB, ρ = 20 g/L), 1.4 M NaCl, 0.1 M Tris, and 20 mM EDTA, pH 8] to each sample (300 mg) and incubating the mixture at 65 °C for 30 min. After incubation, mixtures were centrifuged at 12000*g* for 10 min. The supernatant (500 μL) was transferred into another tube and mixed with 200 μL of chloroform for 30 s. After 10 min of centrifugation, 400 μL of the aqueous phase was transferred into a new vessel, mixed with 800 μL of precipitation buffer (CTAB ρ = 5 g/L, 40 mM NaCl), incubated for 1 h at room temperature, and centrifuged at 12000*g* for 10 min. The buffer was discarded, the precipitate was dissolved in 350 μL of 1.2 M NaCl, and mixed with 350 μL of chloroform for 30 s. After 10 min of centrifugation, 300 μL of the aqueous phase was mixed with 180 μL of 2-propanol in a new vessel and centrifuged for 10 min. 2-Propanol was discarded, and the DNA pellet was mixed with 500 μL of ethanol for 30 s. The ethanol was subsequently discarded, and the pellet was dried at room temperature and finally dissolved in 100 μL of H_2O. DNA concentrations were determined spectrophotometrically at 260 nm using a UV/vis spectrometer (Kontron, Neufahrn, Germany) and adjusted to 20 ng/μL.

Quantification of Bt 176 Corn. Quantification of transgenic maize DNA was performed via real-time PCR using an ABI Prism 7700 Sequence Detection System from Applied Biosystems in combination with two methods developed for determination of GMO contents in foods.

Method A. The GMO content was determined using a commercially available quantification kit (GMO*Quant* Maximizer Bt176 Corn, Lot no. MM: 0040202, GeneScan Europe, Bremen, Germany). This kit included two ready to use master mixes designed for the amplification of a *Zea mays* specific reference sequence, a 79 bp fragment of the high mobility group (*HMGa*) protein genes, and a 104 bp fragment of the synthetic *cryIA(b)* gene encoding the δ-endotoxin of *Bacillus thuringiensis* (*36*). In addition to the FAM-labeled TaqMan probes for the detection of the target sequences, both master mixes also contain a VIC-labeled internal positive control system, allowing the discrimination between true target negatives from a possible inhibition of the PCR. Standard curves were generated by triplicate analysis of the delivered DNA standards.

Method B. The second real-time PCR method used for determination of GMO contents detected a 84 bp fragment of the zein (Z*e1*) gene as reference and a 68 bp fragment of the 35S-CaMV promoter, which has been used in the transgenic maize lines Bt176, Bt11, Mon810, and T25 (*25*). This multiplex PCR system reduced pipetting and heterogeneity bias using a VIC-labeled probe for the detection of the reference gene and a FAM-labeled probe for the transgene. Standard curves were generated by triplicate analysis of DNA dilutions (50−0.05 ng/μL) extracted from Bt176 maize.

RESULTS AND DISCUSSION

Particle Size Distributions. Corn flour, meal, and grits are commonly used to prepare a wide range of products, such as

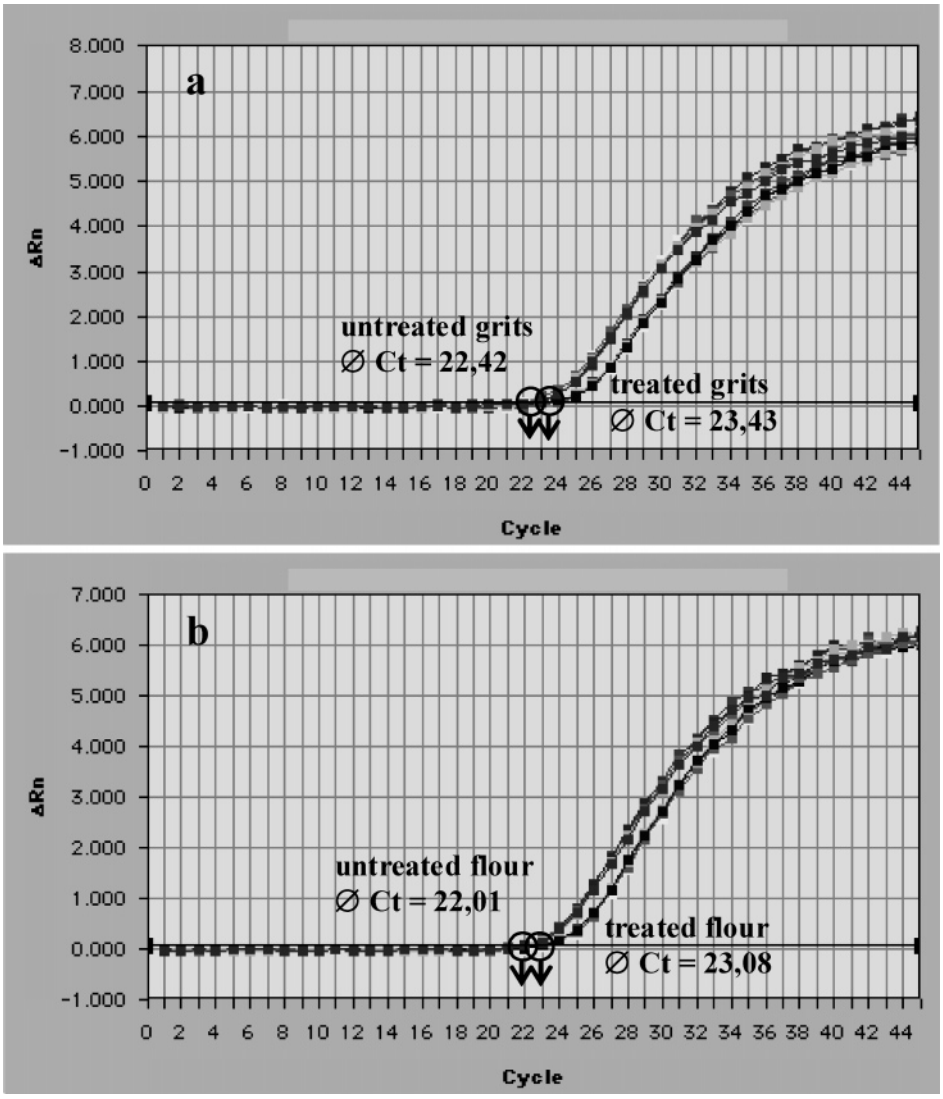

Figure 5. Amplification plots of a 79 bp fragment of the *HMGa* gene in DNA from untreated and heat-treated (120 min) milling products from Bt176 corn (method A). (**a**) Coarse corn grits and (**b**) corn flour. ΔRn = normalized reporter fluorescence; Ø C_t = average threshold cycles.

snacks and bread mixes. Appropriate particle size distributions of the meal and flour mixtures add important functional characteristics to the products and are generally obtained by recombining the previously sieved fractions (*5*, *37*). Sieves used for the preparation of the maize fractions in this study were chosen to mimic particle size ranges commonly used for industrial purposes (*4*). The obtained products were characterized using a laser diffraction system. **Figure 1** illustrates the cumulative distributions of the particle sizes. The experimentally determined median particle sizes (x_{50} values) confirm that a clear separation of fractions with different degrees of comminution could be achieved.

Extraction of DNA from the Milling Fractions. The DNA yields from the different milling fractions were compared using extraction protocols based on DNA-binding silica columns (Wizard method) and on the use of CTAB/chloroform. Both methods have been described as highly appropriate for GMO analysis in foods (*38*) and are used in reference analysis protocols by governmental authorities (*33*, *35*).

Results listed in **Table 2** demonstrate that in both cases, DNA yields increased with an increasing degree of comminution of the milling fraction. This may be explained by the fact that smaller particles offer a larger exposure surface to extraction reagents. The shift in the DNA concentrations of the extracts from coarse corn grits to fine flour was more pronounced when using the CTAB method.

These observations indicated that relative GMO quantifications might be significantly distorted if the analyzed food contains fractions with different particle size distributions. Especially if the presence of GMO is limited to one of the fractions, proportions of DNA extracted from both fractions would not reflect the actual weight proportions of GM material contained in the food sample.

GMO Quantification in Mixtures of Different Particle Size Fractions. Mixtures of coarse grits and flour from conventional and transgenic corn (**Table 1**) were prepared to evaluate the influence of particle size compositions on GMO quantification results. To avoid sampling errors, especially due to variations in homogeneity, the preparation of the sample mixes was performed directly in those reaction vessels in which the DNA extraction was carried out. The proportion of Bt 176 corn in the sample mixes was set to 1% (w/w) to assess the degree of the expected effects at a level of practical interest for the labeling of the presence of GMO in foods or food ingredients. The relative content of transgenic corn DNA in the extracts was determined using a commercially available kit (method A: GMO*Quant* Maximizer Bt 176 Corn, GeneScan Europe) for the quantification of GMO via real-time PCR. The kit consists

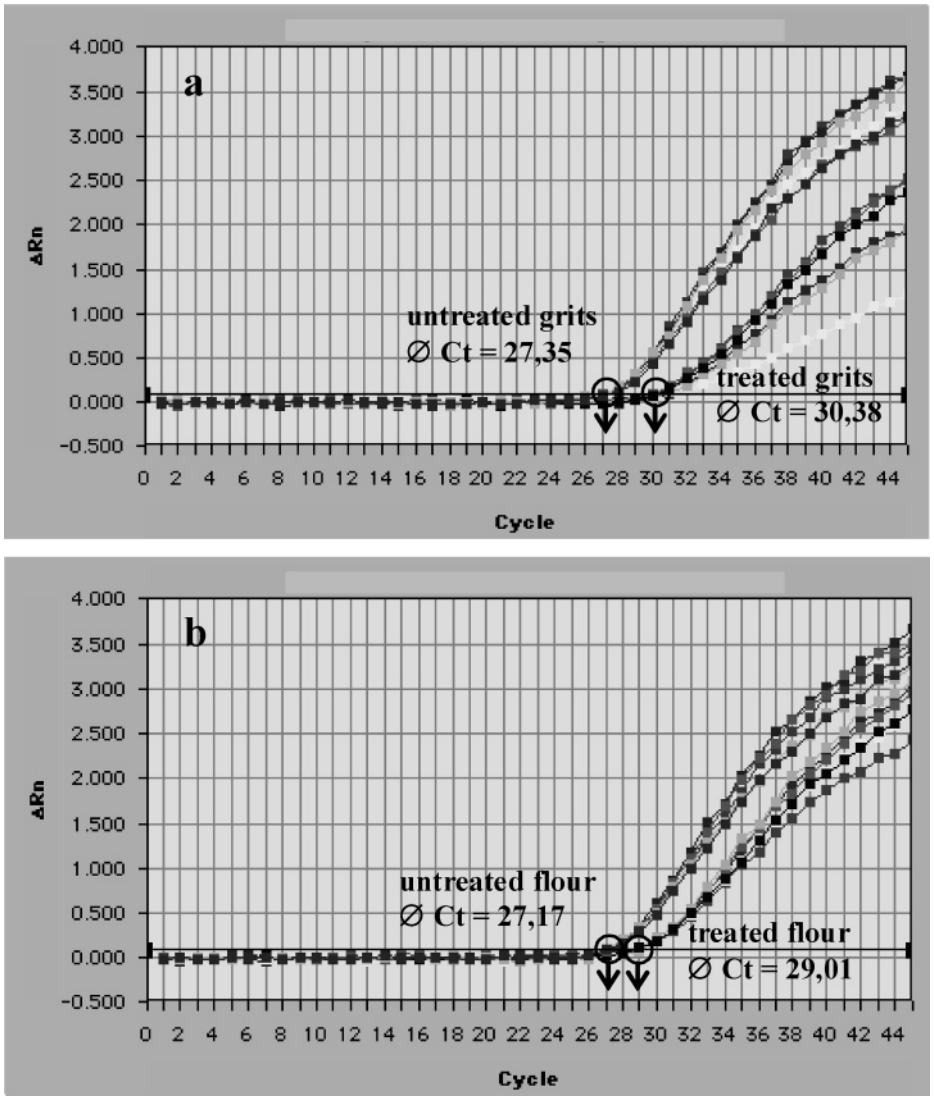

Figure 6. Amplification plots of a 104 bp fragment of the *cryIA(b)* gene in DNA from untreated and for heat-treated (120 min) milling products from Bt176 corn (method A). (**a**) Coarse corn grits and (**b**) corn flour. ΔRn = normalized reporter fluorescence; Ø C_t = average threshold cycles.

of two separate quantitative detection systems, one for the transgenic construct and the other for the maize specific *HMGa* gene, which is used as reference for normalization. In both cases, detection relies on the use of FAM-labeled TaqMan probes.

Figure 2 shows the amplification plots obtained by duplicate analysis of four extracts from each sample mixture, which had been prepared using the Wizard method. The amplification of the reference gene (**Figure 2a**) showed a nearly constant C_t value of 23.0 cycles for all samples, due to the constant amounts of maize DNA used in each reaction. However, when analyzing the *cryIA(b)* gene in mixes containing conventional corn grits fractions and in mixes containing conventional corn flour fractions (**Figure 2b**,**c**, respectively), the C_t values significantly varied depending on the composition of the sample mixture. Extracts from mixes 1 and 4, containing transgenic maize in the form of grits, delivered higher C_t values than mixes 2 and 3, which contained transgenic maize in the form of flour. This is in accordance with the previously described effect of particle size distributions on the effectiveness of DNA extraction and the resulting higher DNA yields from the flour fractions.

The differences between the C_t values of mixes 1 and 4, both containing transgenic maize in form of grits, can also be explained by the improved DNA yield from the isogenic maize flour fraction in mix 4. The DNA extract of mix 4 contained more DNA from conventional maize than mix 1 but equal amounts of DNA from transgenic maize. As DNA concentrations of all extracts were subsequently adjusted to 20 ng/μL prior to analysis, the extract from mix 4 had to be diluted stronger than that from mix 1 due to its higher overall DNA concentration. This resulted in a reduced proportion of DNA from the transgenic maize grits and consequently in higher C_t values for the transgenic target sequences. Analogous reasoning can be used to explain differences between C_t values of mixes 2 and 3, both containing transgenic maize in form of flour.

These phenomena were clearly reflected in the results obtained by quantitative analysis of the sample mixes. As shown in **Figure 3**, significant distortions of the predicted GMO contents were observed in all mixes containing heterogeneous transgenic and conventional corn fractions. The quantification of mixes containing coarse grits and flour (mixes 3 and 4) resulted in a significant over- and underestimation of the adjusted GMO proportions. In contrast, the quantification of mixes 1 and 2 containing fractions with similar particle size distributions delivered the expected results and was unaffected by the DNA extraction methodology used.

Distortions of quantification results were lower but still substantial if DNA was extracted using the Wizard extraction method as compared to the CTAB/chloroform method. This may

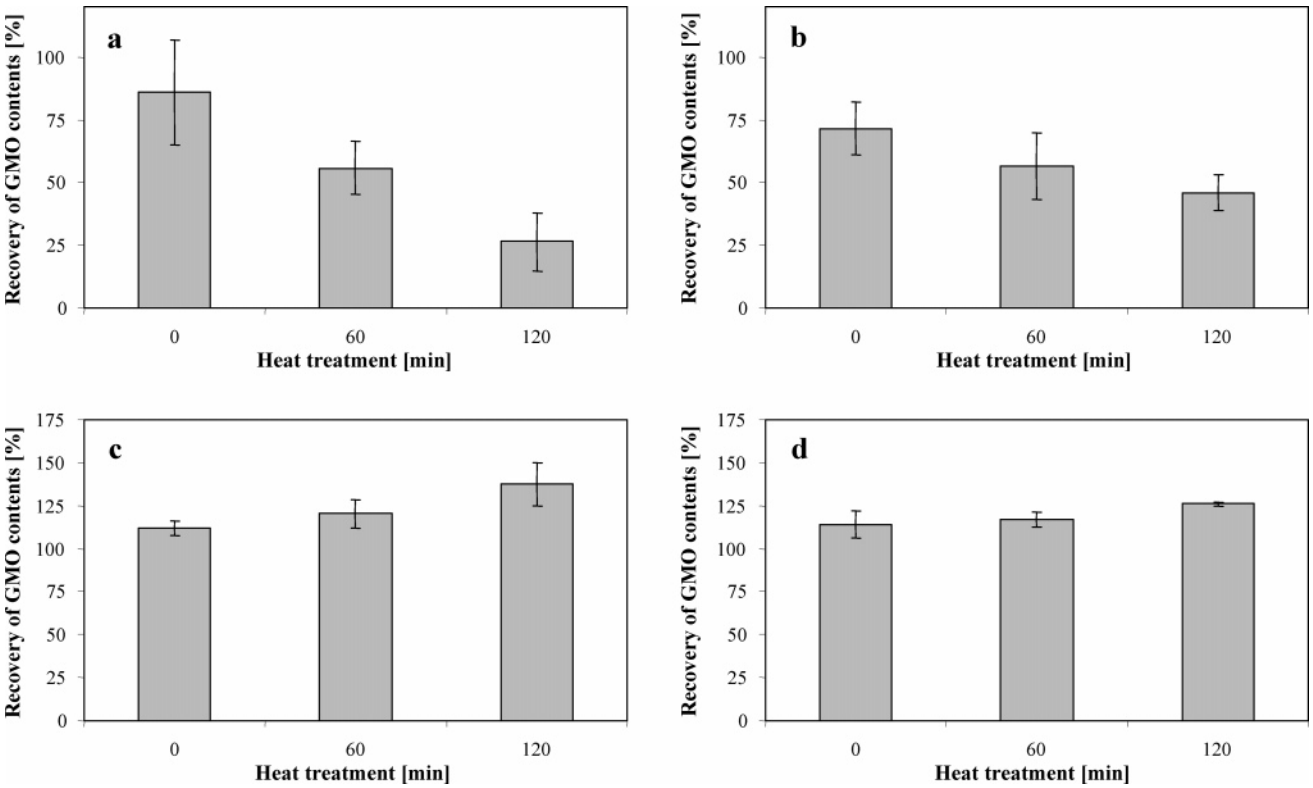

Figure 7. Effect of thermally induced DNA degradation on the recovery of GMO contents. Quantification of (**a**) coarse corn grits and (**b**) corn flour samples via method A; amplicon lengths: *cryIA(b)* gene, 104 bp; *HMGa* gene, 79 bp ($\Delta l_A = -25$ bp). Quantification of (**c**) coarse corn grits and (**d**) corn flour samples via method B (*21*); amplicon lengths: 35S CaMV promoter, 68 bp; zein (*Ze1*) gene, 84 bp ($\Delta l_B = +16$ bp). Error bars indicate standard deviations of the means.

be ascribed to the lower variation of the extraction efficiency of the Wizard method when isolating DNA from flour and coarse grits, in comparison to the variation of the extraction efficiency shown by the CTAB/chloroform method (**Table 2**).

Recovery of Target Sequences at Different Degrees of DNA Degradation. A degradation of DNA in coarse grits and in flour from maize Bt-176 was induced by heat treatment for 60 and 120 min at 100 °C. As shown in **Figure 4**, electrophoretic analysis of the DNA isolates (Wizard extraction) pointed in both cases to a gradual breakdown of DNA in the course of the heating process. The degradation of DNA in the coarse grits matrix seemed to have reached a higher extent than that observed in the flour matrix.

The influence of DNA degradation on the relative quantification of recombinant target sequences was evaluated using a commercially available kit (method A). Standard curves covering a range of high copy numbers for the reference *HMGa* gene (160−81920 copies) and a range of low copy numbers for the transgene (10−5120 copies), were generated using the enclosed external quantification standards. As the analyzed samples consisted of 100% GMO-derived material, DNA concentrations of the isolates were adjusted to 20 ng/μL for the analysis of the reference gene and were further diluted with elution buffer (1:100) for the analysis of the transgene. This was necessary to allow the analysis of the samples using standard curves covering different ranges of copy numbers. Adjustment of DNA concentrations in the isolates also permitted the use of constant amounts of template DNA in each amplification reaction, thus allowing a direct comparison of the C_t values of the target sequences at the different stages of treatment. For each heating stage, three independent DNA extracts were analyzed to allow the acquisition of representative results. Each reaction was run in duplicate.

Figures 5 and **6** illustrate the amplification plots of the target sequences before and after heat treatment for 120 min. The application of 100 ng of template DNA in each amplification reaction resulted in nearly constant C_t values for each target sequence within the respective stages of treatment. However, the comparison of the amplification plots from both target sequences indicated a significant shift of the curves toward higher C_t values after thermal treatment. This effect can be explained by the breakdown of DNA during heat treatment, which continuously reduces the proportion of amplifiable target sequences.

Degradation rates of target sequences are expressed as differences (ΔC_t) between mean C_t values within 120 min of thermal treatment. Taking into account the nearly consistent relative standard deviations of 1% observed for the measurements of the mean C_t values, the degradation rates evidenced a stronger fragmentation of the target fragment used for the detection of the *cryIA(b)* gene. This was observed regardless of whether DNA was extracted from coarse grits or flour. For instance, the analysis of coarse grits delivered a $\Delta C_t = 3.03$ for the 104 bp long target sequence of the transgene (**Figure 6a**), which was statistically significantly higher than the $\Delta C_t = 1.01$ determined for the 79 bp long target sequence of the reference gene (**Figure 5a**). Degradation rates of the target sequences in the flour matrix (**Figures 5b** and **6b**) were not as pronounced as in the coarse grits matrix (ΔC_t *cryIA(b)* $= 1.84$; ΔC_t *HMGa* $= 1.07$). These results confirmed the observations made in the electrophoretic analysis of the sample extracts, which pointed to a reduced fragmentation of DNA in the flour fraction.

Effects of Length Differences between Recombinant and Reference Target Sequences on Relative Quantification Results. The increased sensitivity of the transgenic target toward

stress conditions was ascribed to the length difference between the amplified fragments, since the detection of the synthetic *cryIA(b)* gene was performed targeting at a longer DNA fragment than that used for the detection of the maize specific *HMGa* gene ($\Delta l_A = -25$ bp). As illustrated in **Figure 7a**,**b**, disproportional degradation rates between transgenic and reference target sequences resulted in a continuously increasing distortion of relative quantification results. Despite the observed standard deviations reflecting the uncertainties arising from triplicate DNA preparations and duplicate measurements via real-time PCR, the average recovery values obtained show a significant underestimation of GMO contents in the samples heat-treated for 120 min. Distortions were more pronounced in the samples of heat-treated coarse grits. This reflected the stronger degradation of DNA in the coarse grits fraction that had been observed in the electrophoretic analysis as well as in the respective amplification plots.

To confirm that the observed distortion of relative quantification results was a consequence of disparities in the degradation rates of recombinant and reference target sequences, a second real-time PCR assay (*25*) (method B) was used. The assay was designed for the detection of a 68 bp fragment of the 35S-CaMV promoter and an 84 bp fragment of the maize specific zein (*Ze1*) gene. In contrast to the previously applied commercial kit, the targeted sequence for the reference gene was in this case longer than that for the transgene (length difference $\Delta l_B = +16$ bp). Reactions were carried out in a multiplex format using FAM-labeled and VIC-labeled TaqMan probes. Standard curves were generated on the basis of triplicate analysis of self-prepared quantification standards, consisting of a dilution series of DNA extracted from maize Bt-176. The use of DNA standards covering a concentration range between 50 and 0.05 ng/μL delivered curves of very good linearity ($R^2 > 0.99$) and amplification efficiency (97.2 and 97.1%, respectively).

In this method, the use of a longer sequence for the detection of the reference gene ($\Delta l_B = +16$ bp) resulted in an increased susceptibility of this target toward stress conditions. Thus, the application of this method for the analysis of the DNA isolates from heat-treated samples resulted in an increasing overestimation of GMO contents (**Figure 7c**,**d**). The degree of distortion of the relative quantification results was lower than that observed in the previous method, due to the smaller absolute length difference between the targeted sequences for the recombinant DNA and the transgene. Again, distortions were more pronounced in the samples of heat-treated coarse grits.

These results demonstrate how common food manufacturing practices, such as the use of ingredients with different particle size distributions or the application of heating processes, may strongly affect the accuracy of GMO quantification. Distortion of quantification results owing to unequal DNA extraction efficiencies from ingredients with different particle sizes was observed for the two major extraction procedures applied in food analysis. The accuracy of GMO quantifications in thermally treated samples was strongly affected due to disproportional degradation rates of recombinant and reference targets when aiming at sequences of different lengths.

Data gained from the performance assessment of the applied assays deliver important criteria for future method development and for the design of validation studies. Especially in regard to the establishment of standardized international protocols for the quantitative analysis of GMO in foods, validation studies cannot be restricted to the assessment of trueness and precision in (unprocessed) certified reference materials, since such performance parameters may not be unconditionally transferable to results obtained from the analysis of composed and/or processed samples. Therefore, procedures for the validation of quantitative assays for the surveillance testing of products within the food chain must include experiments demonstrating that neither food composition nor processing will result in an alteration of relative quantification results. Future standard protocols for the determination of GMO contents in processed products should target recombinant and taxon specific sequences of nearly equal lengths. Otherwise, the scope of the analytical approaches should be clearly limited to the determination of GMO contents in unprocessed samples.

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