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Use of Quantitative Real-Time PCR To Estimate Maize Endogenous DNA Degradation after Cooking and Extrusion or in Food Products

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Polymerase chain reaction (PCR) is being used increasingly to detect DNA sequences for food quality testing for GM content, microbial contamination, and ingredient content. However, food processing often results in DNA degradation and therefore may affect the suitability of PCR or even DNA sequence detection for food quality assurance. This paper describes a novel approach using quantitative real-time PCR (qPCR) to estimate the extent of DNA degradation. With use of two maize endogenous nuclear sequences, sets of four qPCR assays were developed to amplify target sequences ranging from <100 bp to approximately 1000 bp. The maize nuclear sequences used encode chloroplastic glyceraldehyde-3-phosphate dehydrogenase and cell wall invertase. The utility of the qPCR approach for quantifying the effective concentration of maize DNA that is needed to amplify variable length DNA sequences was demonstrated using samples of maize cornmeal cooked in water for variable times, extrusion products developed using different barrel temperature and torque settings, and a range of food products from supermarket shelves. Results showed that maize DNA was substantially degraded by a number of processing procedures, including cooking for 5 min or more, extrusion at high temperatures and/or high torque settings, and in most processed foods from supermarket shelves. Processing also reduced the effective concentration of DNA sequences capable of directing amplification of the <100 bp assays as well, particularly after popping of popping corn or extrusion at a combination of high temperature and torque settings. The approach for quantifying DNA degradation described in this paper may also be of use in disciplines where understanding the extent of DNA degradation is important, such as in environmental, forensic, or historical samples.

KEYWORDS: ***Zea mays*; extrusion; processing; DNA degradation; polymerase chain reaction**

INTRODUCTION

As DNA detection technologies have developed, in particular, the polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR), DNA-based diagnostics have been used increasingly for food quality testing. PCR diagnostics have been applied widely for detecting microbial contamination (*1*, *2*), food content derived from genetically modified organisms (GMOs, reviewed in (*3*)), and contamination by unintended ingredients such as cereals in gluten-free foods (*4*) and beef or pork (*5*).

The ability to detect the DNA of contaminating material by PCR depends on the quality of the extractable DNA, which in turn reflects the degree of DNA degradation that has occurred, the possibility of DNA forming covalent complexes with food constituents such as proteins, and the presence or absence of PCR inhibitors (*6*). The effect of inhibitors on PCR varies with the specific food matrix and depends on the DNA extraction method applied as well as the thermostable DNA polymerase used in amplification reactions (*1*, *7*). For example, DNA extraction methods for corn kernels as well as the inhibitory effects of mono- and polysaccharide constituents of corn kernels on PCR reactions have been reported (*6*). Likewise, the suitability of different DNA extraction methods for extracting soy DNA from complex foods such as chocolate and biscuits is described in the literature (*8*).

DNA degradation as a result of food processing and its effect on PCR-based sequence detection has been examined (*9*−*13*). In the above examples, DNA degradation from food processing was characterized using conventional PCR with different target length amplicons detected on agarose gels. In contrast to conventional PCR, quantitative real-time PCR (qPCR) has the ability to estimate accurately the amount of material present in a sample that is capable of acting as a template for amplification of the sequence of interest. A number of strategies have been developed for carrying out qPCR (*14*) and methods have been developed for detecting GMO-derived DNA content in foods (for example, refs *15*−*18*). With use of appropriate endogenous sequence controls to quantify DNA added to PCR reactions,

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qPCR has the advantage over conventional PCR of enabling the percent of GMO-derived content in a food or food ingredient to be estimated. Accurately estimating the amount of GMO-derived ingredients in foods is important since many national governments or governing bodies require the labeling of foods that originate from GMOs. For example, European Union (EU) regulations have established a 1% content threshold for the labeling of food, food ingredients, or feed for the presence of adventitious, approved GMO-derived material (summarized in ref *3*). qPCR assays such as the fluorogenic 5′ nuclease (TaqMan) technology that typically amplify small (<100 bp) DNA fragments may also be more appropriate than conventional PCR for detecting sequences in DNA that has been degraded during food processing.

In this paper a novel approach is described for estimating the extent of DNA degradation into different fragment sizes and concentrations in processed maize products using qPCR. Four sets of TaqMan assays ranging in size from <100 to ~1000 bp were developed for each of two endogenous *Zea mays* nuclear genomic sequences. The degradation of DNA as a result of processing by cooking or extrusion, or in maize-containing products taken from supermarket shelves, was then quantified using these assays. The approach we describe for assessing DNA degradation in processed foods therefore provides a method for critically assessing the size range and quantity of DNA fragments available for sequence detection by PCR. Although demonstrated on maize-containing food or foodlike products, this method is of practical significance for any PCR-based diagnostic or sequence detection system that may be affected by DNA degradation. This includes detection of GMO content, ingredients, or microbes in foods but may also see wider application in disciplines such as studies of horizontal gene transfer, ancient DNA, forensic DNA diagnostics, DNA analysis of soils, and examination of microbial cell viability and autolysis.

MATERIALS AND METHODS

Samples. Samples of freshly ground maize cornmeal were made by grinding dry maize kernels (food grade yellow maize, Corson Grain Ltd., Gisborne, New Zealand) in a blender. Immediately after grinding, cornmeal to be used for cooking experiments was chilled on ice to minimize the likelihood of DNA degradation. A range of maize-containing food products, including popping corn, corn-based snack foods, tortillas, canned sweet corn, and frozen sweet corn, were purchased from a local supermarket. A sample of the popping corn was popped in a domestic hot-air-driven popcorn machine.

Heat Treatment of Maize Cornmeal. Subsamples (50 ± 0.5 mg) of freshly ground maize cornmeal were mixed with 100 μL of sterile distilled H_2O. Four replicate subsamples were then heat-treated at 100 °C for 0, 5, 15, or 60 min and then placed immediately on ice to await extraction.

Extrusion. Extruded products for quantitative analysis of DNA content were produced using 320 spec maize grits (Corson Grain Ltd., Gisborne, New Zealand) and water as the starting materials. The ingredients were processed using a Clextral BC21 Twin-screw Extruder (Clextral, Firminy, France) set to give a range of different shear rates and processing temperatures. The screw configuration was 2 × 50 mm forward coarse, 2 × 50 mm forward medium, 1 × 50 mm forward fine, 1 × 25 mm reverse fine, 2 × 500 mm forward fine, and 1 × 25 mm reverse fine. Experiments were carried out at barrel temperatures of 60 and 170 °C and at six torque settings corresponding to approximately 6, 12, 18, 24, 30, and 36 Nm. The torque was controlled by adjusting the rate of water addition between 0.5 and 2.5 L/h and feed rate of the grits between 3 and 8 kg/h. The screw speed was constant for each temperature, 150 rpm at 60 °C and 400 rpm at 170 °C. Conditions were changed systematically to reduce equilibration times within the extruder. At each temperature, the torque was increased incrementally to the maximum setting used and then decreased incrementally to the minimum setting. The order of samples was (temperature/torque) as follows: 60 °C/6 Nm, 60 °C/12 Nm, 60 °C/18 Nm, 60 °C/24 Nm, 60 °C/30 Nm, 60 °C/36 Nm, 60 °C/36 Nm, 60 °C/18 Nm, 60 °C/6 Nm; and 170 °C/6 Nm, 170 °C/12 Nm, 170 °C/18 Nm, 170 °C/24 Nm, 170 °C/30 Nm, 170 °C/36 Nm, 170 °C/36 Nm, 170 °C/18 Nm, 170 °C/6 Nm.

DNA Extraction. Each supermarket food product sample was homogenized in a blender and three subsamples were removed for DNA extraction. Each extrusion sample was ground and one subsample was removed for DNA extraction. Subsample sizes were 50 ± 0.5 mg for dry products (extrusion products, popped and unpopped popping corn, snack foods, and tortillas). For blended wet products (canned and frozen corn) 100 ± 2 mg was used. For each cooking time sample, four replicate maize cornmeal samples (50 mg of cornmeal + 100 μL of H_2O) were heat-treated and extracted.

DNA extraction was carried out using a Nucleon PhytoPure Genomic DNA Extraction Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Extractions included the optional spin to clarify the retained aqueous phase after the resin/chloroform step. DNA from each sample was resuspended in 400 μL of 5 mM Tris, 0.5 mM EDTA buffer, pH 8.0.

The quantity and extent of degradation of extracted DNA was examined by electrophesis through 1% agarose containing 1X TBE (*19*). DNA extractions were examined with and without digestion with RNase A (Qiagen, Germantown, MD), carried out according to Sambrook et al. (*19*). Standards electrophoresed alongside the samples included 1 Kb Plus DNA ladder (Invitrogen, Carlsbad, CA) as well as maize tissue calibration standards. Following electrophoresis, gels were stained with SybrGreen I (Invitrogen, Carlsbad, CA) and nucleic acids were visualized by fluorescence using a GelDoc 2000 with Quantity One software (BioRad, Hercules, CA). Using the "highlight saturated pixels" option, exposure parameters were set so that band intensities did not saturate the camera.

Preparation of Maize Leaf Total DNA Concentration Standards. Standard curves for each of the PCR assays were constructed using DNA extracted from young maize leaf tissue. After grinding of approximately 100 mg of fresh maize leaf tissue in liquid N_2, DNA was extracted as described above. The concentrations of maize DNA in the extractions were determined by comparing a set of DNA concentration standards prepared using Fluorescence Standard Calf Thymus DNA (GE Healthcare, Piscataway, NJ) that was mixed with 100 ng/mL Hoechst dye (Sigma, St. Louis, MO). Fluorescence signals were compared using a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). The maize leaf DNA was then diluted in water to concentrations ranging from 5 pg/μL to 5 ng/μL. Calibration curves for real-time PCR assays were generated by adding 5 μL of these dilutions to the real-time PCR assay mixtures, giving seven DNA concentrations ranging from 25 to 25000 pg/PCR reaction.

Primer Design. Oligonucleotide primers and probes for real-time PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA) to amplify four differently sized products from each of two *Zea mays* endogenous nuclear genes. The genes were chloroplast glyceraldehyde-3-phosphate dehydrogenase (G3PD, Genbank accession M18976) and cell wall invertase (INCW2, Genbank accession AF050128). For each sequence, a single antisense primer and adjacent probe were combined with each of four different sense primers to yield amplicons of four different sizes (**Figure 1**). Primer and probe sequences are presented in **Table 1**. Primers were synthesized by MWG-Biotech AG (Germany) while the dual-labeled probes were synthesized with 5′ 6FAM and 3′ BHQ1 (Black Hole Quencher-1) dual fluorescent labels (GeneWorks, Melbourne, Australia).

Quantitative Real-Time PCR Amplifications. Real-time PCR was carried out using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using fluorescent probe-based technology. This approach allows PCR products to be quantitated in real time by measuring the increase of fluorescence of the reporter dye attached to the 5′ end of the probe following its release by the processive 5′ nuclease activity of Taq polymerase. Optimal primer and probe concentrations were determined to obtain the lowest C_t and highest fluorescent intensity (ΔRn) values. The range of primer concentrations

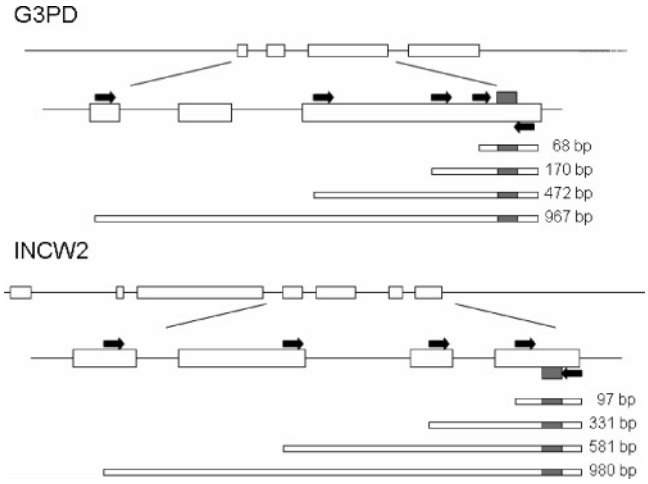

Figure 1. Organization of the *Zea mays* genes G3PD and INCW2. Exon sequences to which primers have been designed are indicated. The locations of primers (black arrows) and probes (gray boxes) for amplification of PCR products of variable length by real-time PCR are also shown.

tested was 50−900 nM and the range of probe concentrations tested was 25−200 nM.

Real-time PCRs on samples, calibration curve standards, and controls were carried out in 25 μL that contained 1X TaqMan Universal Mastermix (Applied Biosystems, Foster City, CA), dual-labeled probes (100 nM), and primers (300 nM each, except for primers G3PD_F472 and G3PD_F967, which were added at 900 nM each). Sample DNA or diluted standard DNA was added to PCR reactions in 5 μL volumes. Assays were carried out in 96 well optical PCR plates sealed with optical film (Applied Biosystems, Foster City, CA). The amplification protocol used is different from the standard two-stage TaqMan protocol and included an initial incubation at 50 °C for 2 min for AmpErase uracil-*N*-glycosylase (UNG) activity and a 95 °C incubation for 10 min for AmpliTaq Gold activation, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min.

Samples were analyzed with each of the eight PCR assays (the four INCW2 and four G3PD assays) in separate runs. For each of the eight assays, two replicate PCR reactions were carried out from each DNA extraction. Calibration curves for maize DNA were developed by running each of the eight PCR assays in duplicate using seven maize leaf DNA quantity standards (25 pg, 75 pg, 250 pg, 750 pg, 2.5 ng, 7.5 ng, and 25 ng) per PCR reaction. No template controls (NTC) were included in each run in duplicate. The samples, calibration standards, and NTC were randomized in 96 well plates, and the same randomization was used for all eight assays. Heat-treatment and food-product samples were assessed in one set of 96 well plates; a separate set of 96 well plates was used for the extrusion samples. The threshold cycle parameters (C_t values) were recorded for each assay using the default thresholds calculated by Sequence Detection Systems 1.9 software (Applied Biosystems, Foster City, CA).

Statistical Analysis. A calibration curve was first estimated for each of the eight assays (runs) by regressing the C_t values for the maize leaf DNA standards on the log(quantity). Regressions for the eight heat-treatment and food-sample assays were carried out together, as were the regressions for the eight extrusion sample assays. Maize DNA standard amounts for which amplification was not detected were excluded before calibration curve regressions were carried out. C_t values for the samples were converted to estimated maize DNA amounts using the equations derived from these regressions. Estimated DNA amounts were analyzed separately for each assay, and separately for the heat treatment or food samples and extrusion samples (16 analyses in all). The efficiency (E) of a single PCR cycle in the exponential phase of qPCR assays was calculated using the equation $E = 10^{(-1/\text{slope})}$ (*20*).

Estimated DNA is an indirect estimate of the number of template molecules in the sample. As such, the DNA estimates have many of the properties of count data. Therefore, methods appropriate for count

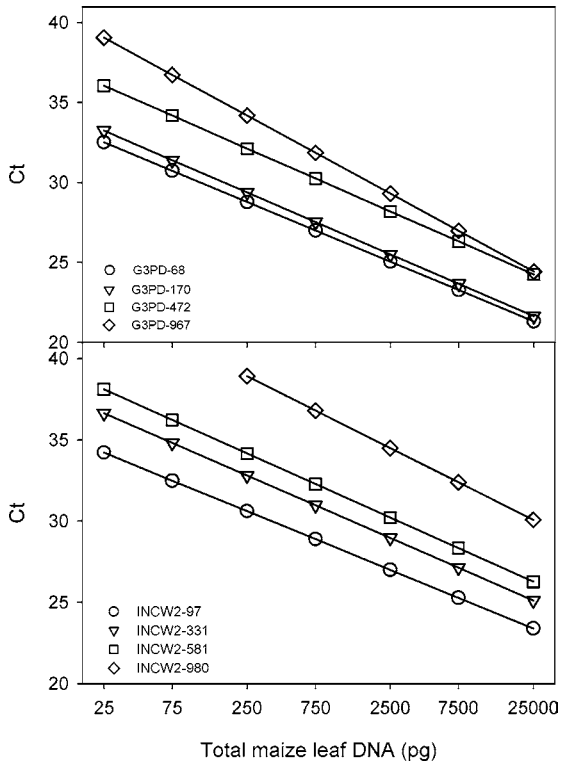

Figure 2. Examples of calibration curves for G3PD (top) and INCW2 (bottom) sequence-based real-time PCR assays of variable length. The C_t values obtained are plotted against the amount of log(total maize leaf DNA) added per real-time PCR reaction (in picograms).

data were used to analyze these data, with adjustments. The standard approach for analyzing count data is to use a generalized linear model (GLM) with a logarithmic link and a Poisson error (*21*). The data were initially examined using a simple Poisson GLM, which included just a factor for the differences between samples. For the heat treatment or food samples, examination of residuals from these analyses strongly suggested that the simple Poisson model did not adequately account for the patterns of variability in the data, so the data were then examined with a hierarchical GLM (HGLM, *22*) again using Poisson errors and a logarithmic link. The HGLM allows random factors to be included in the analysis and is a more general approach than the commonly used generalized linear mixed model (GLMM, *23*). For the heat treatment and food samples, the dispersion (roughly, random variation) was modeled separately for each product. This was not necessary for the extrusion data. Also, a random effect (with a normal distribution) was included for the heat treatment or food sample extractions or replicate runs of extruder settings. Examination of residuals after fitting these models indicated that the patterns in the variance had been adequately explained. The estimated dispersion component for the extractions or runs were also large for most of the assays, indicating that there was more variability between extractions from a product or between runs of an extruder setting than between the duplicates from the same extraction or run. This was as expected. Therefore, these models were used for the final analyses of the data.

Confidence limits for the estimated mean DNA for each product were calculated by using the estimated standard errors for the estimates on the log scale, and then back-transforming. The residual degrees of freedom from the analyses were used as approximate degrees of freedom associated with the standard errors. Comparisons between products within any assay were made within the HGLM analysis, using contrasts, and a significance level of 5%. Comparisons between assays were made informally, using graphs.

For several of the assays, the C_t values on all subsamples indicated that no target sequence was detected. The data for each such product were excluded from the analysis, as described above, since there was no variability between their subsamples, and including the data within the analysis could have led to bias. However, ad hoc comparisons

Table 1. Sequences of Oligonucleotide Primers and Probes Used in Quantitative Real-Time PCR Assays To Detect *Zea mays* Chloroplast Glyceraldehyde-3-phosphate Dehydrogenase (G3PD) and Cell Wall Invertase (INCW2) Sequences

oligonucleotide	strand	sequence (5–3)	amplicon (bp)
G3PD_R	antisense	GCAGGAGGCGTTGCTGAT	
G3PD_Pf [a]	sense	CCAGTACAACCCCGACGAGCCCA	
G3PD_F68	sense	CCTACGTCGTCGGCGTTAAT	68
G3PD_F170	sense	AGGGCACCGGCGTCTT	170
G3PD_F472	sense	GACGGAGGCGAAGCTGAA	472
G3PD_F967	sense	TCCATGCTCTCCGCTACCA	967
INCW_R	antisense	TTCAGGCCCCGTTCATGA	
INCW_Pf [a]	antisense	CTTCATCTCCCACGCGGTGAGACG	
INCW2_F97	sense	GCTCGCCTCTACGTGTTCAAC	97
INCW2_F331	sense	CATCTCGAACGGCAAGATATCC	331
INCW2_F581	sense	ATGTGCACCGACCCTACCAA	581
INCW2_F980	sense	CAAAGTCGGTCACTCTCAGGAA	980

[a] Fluorescent probe.

between the products with no detectable target DNA and the others could be made. If the confidence limits for a sample with a non-zero-estimated mean DNA content did not include zero, then that product could be said to have significantly more DNA than 0, at least at the 95% level. Confidence limits for the products or extruder settings with zero-estimated target DNA were made by estimating the confidence limit for a count of zero using the method of Daly (*24*) and then translating these counts into DNA content by multiplying by the *Zea mays* DNA C-value of 1C = 2.73 pg (*25*). A similar approach was used for a very few cases where the estimated DNA was very small and estimates of the standard errors could not be estimated asymptotically within the analyses.

Calibration regressions and the main statistical analyses were carried out with GenStat (*26*).

RESULTS

Design of Variable Length Real-Time PCR Assays. Real-time PCR assays were designed to produce amplification products that ranged in length from small (<100 bp) to approximately 1000 bp for maize sequences encoding glyceraldehyde-3-phosphate dehydrogenase (G3PD) and cell wall invertase (INCW2) (**Figure 1** and **Table 1**). Primers and probes were designed to bind to exon (coding region) sequences of each gene. A single right primer sequence and a single probe sequence were used for the four assays for each maize gene. Different length amplicons were produced by varying the left primer sequence. Assay conditions were chosen so that identical thermal cycling conditions could be used for all eight assays.

Characterization of the Standard Curves. Standard curves were constructed for each of the eight real-time PCR assays using dilutions of total DNA extracted from young maize leaves (**Figure 2**). The haploid DNA content (1C) of the *Zea mays* genome is 2.73 pg (*25*). Therefore, 25 pg, the smallest amount of total maize DNA used in the standard curves, contains less than 9.15 copies of the maize genome since both mitochondrial and chloroplast DNA are present in total DNA extractions.

Standard curve parameters showed the influence of amplicon length on the sensitivity and efficiency of real-time PCR assays for detecting the target sequences in maize DNA extracted from leaf tissue when product lengths varied from <100 bp to approximately 1000 bp (**Table 2**). PCR assays that amplified short products were more sensitive than assays that amplified longer products. Furthermore, amplification of the INCW2-980 product was not detected when only 25 or 75 pg of maize leaf DNA was added to reactions. The standard curves indicated that the assays that amplified short PCR products were more efficient than the assays for the long amplification products. Overall, lower C_t values were obtained for the G3PD assays than the INCW assays (**Figure 2**).

Table 2. Parameters for Calibration Curves for Real-Time PCR Assays That Amplify Various Length Target Sequences from the *Zea mays* G3PD and INCW2 Genes[a]

assay	C_t intercept (se)	slope (se)	R^2	efficiency
G3PD-68	37.72 (0.39)	−3.73 (0.13)	99.6	1.854
G3PD-170	38.64 (0.39)	−3.87 (0.13)	99.5	1.813
G3PD-472	41.56 (0.39)	−3.94 (0.13)	99.5	1.794
G3PD-967	45.89 (0.39)	−4.88 (0.13)	98.2	1.603
INCW2-97	39.26 (0.39)	−3.61 (0.13)	98.8	1.892
INCW2-331	42.01 (0.39)	−3.84 (0.13)	97.6	1.821
INCW2-581	43.63 (0.39)	−3.95 (0.13)	98.4	1.791
INCW2-980	49.50 (0.73)	−4.42 (0.21)	99.3	1.684

[a] Standard errors (se) for C_t intercept and slope are presented. Efficiency of a single PCR cycle in the exponential phase of qPCR was calculated using the equation $E = 10^{(-1/\text{slope})}$.

Electrophoretic Assessment of DNA Extractions. The quantity and quality of DNA recovered after extractions on all samples was assessed by electrophoresis through agarose gels to reveal the presence of high molecular weight versus degraded DNA. **Figure 3** shows a typical gel containing calibration standards and samples that have undergone experimental food processing steps (cooking and extrusion). Electrophoretic migration of extracted DNA clearly showed the effects of processing on DNA degradation, which is particularly apparent from examining the presence of the high molecular weight DNA band versus a smear of lower molecular weight DNA, as well as by examining the relative intensity of the staining.

DNA Degradation during Heat Treatment of Cornmeal. The usefulness of the G3PD and INCW2 real-time PCR assays for assessing DNA degradation in cooked and processed foods was demonstrated by analyzing DNA extracted from samples of cornmeal mixed with water that were heat-treated in a dry block at 100 °C for various times (**Figure 4**). The G3PD-68 and INCW-97 assays showed that DNA was recovered quantitatively in extractions from samples that were cooked for 5 or 15 min since the estimated DNA content of those extractions was not significantly different ($p > 0.05$) from the DNA content of extractions from the uncooked cornmeal and water mixture. In contrast, less DNA was recovered after 60 min of cooking, based on the results of the INCW-97 and G3PD-68 real-time PCR assays. This was especially apparent from the INCW-97 results. The reduced recovery may indicate that the DNA was degraded by cooking to such an extent that fewer target molecules for these short products were available, or that the ability to recover DNA from the cooked cornmeal and water matrix was reduced after 60 min of heating at 100 °C.

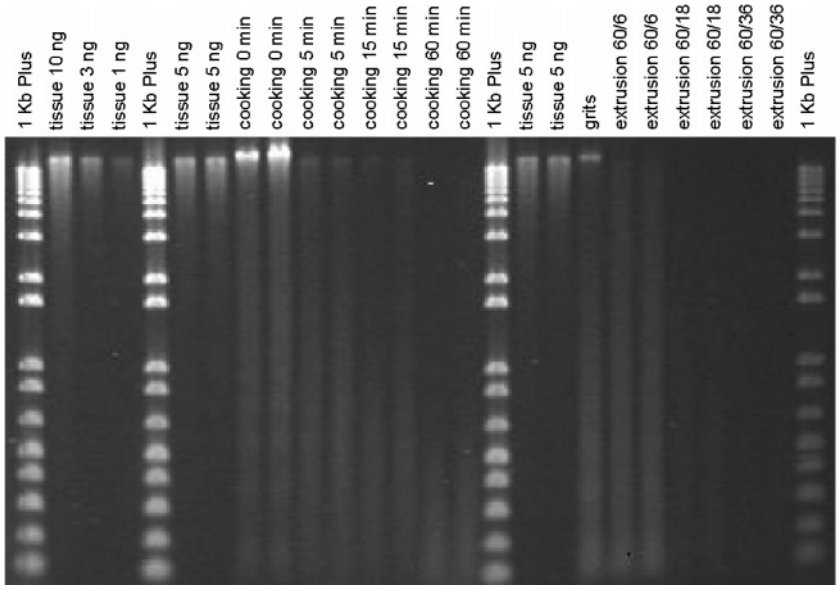

Figure 3. Electrophoretic examination of the relative quantities and degradation of DNA recovered after extraction of cooked cornmeal and extrusion samples, compared with known quantities of maize DNA extracted from leaf tissue. Cooking times and extrusion parameters (temperature in °C and torque in Nm) are included in lane descriptions. DNA ladder lanes are labeled (1 Kb Plus).

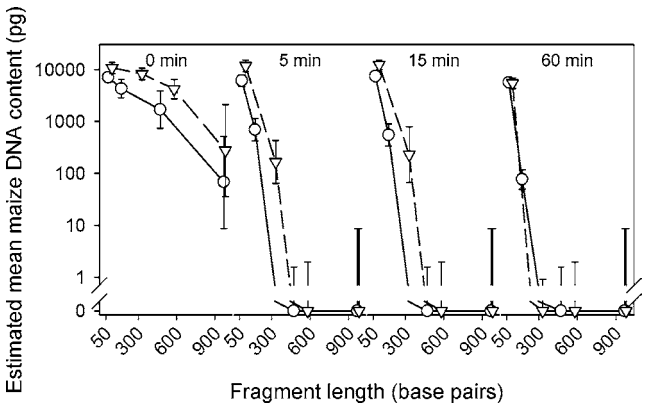

Figure 4. Real-time PCR estimation of mean maize DNA content for variable length target sequences in cornmeal and water mixtures after 0, 5, 15, and 60 min of cooking at 100 °C. The mean DNA content (in picograms) of aliquots of extractions representing 625 ng of maize cornmeal in the starting material was estimated using variable length real-time PCR assays for the G3PD (triangle symbols) and INCW2 (circle symbols) sequences. Each point represents the estimated DNA amounts derived from eight real-time PCR reactions. Error bars indicate 95% confidence intervals.

When analyzing DNA that has been degraded to some extent, each quantitative real-time PCR assay that amplifies a different length product (e.g., 97 bp versus 980 bp) will estimate the amount of DNA in the PCR reactions that is at least as long as the amplicon length and contains undegraded target sequences. Examination of the apparent DNA recovery from the uncooked maize when estimated using PCR assays for the various amplicon lengths (**Figure 4**) shows that the DNA extracted from the freshly ground cornmeal has undergone considerable degradation. For example, the INCW-97 assay estimated that about 10.7 ng of DNA was added to each qPCR reaction, while the INCW-980 assay found only approximately 0.27 ng of DNA. Likewise, the G3PD-68 assay estimated about 7.1 ng of maize DNA while the G3PD-967 assay detected about 0.067 ng. Intermediate estimates of the effective DNA content were obtained from uncooked maize cornmeal extractions using the INCW-581 (4.2 ng) and G3PD-472 assays (1.7 mg).

Cooking markedly reduced the effective DNA concentration for all assays longer than the INCW-97 or G3PD-68 assays (**Figure 4**). After cooking for 5, 15, or 60 min, no maize DNA was detectable with the INCW-581, INCW-980, G3PD-472, or G3PD-967 assays. As noted above for the 5 and 15 min of cooking samples, DNA recovery in extractions was quantitative, as determined by the INCW-97 and G3PD-68 assays. Therefore, the reduction in the concentration of DNA that was detectable using the INCW-331, INCW-581, or INCW-980 and the G3PD-170, G3PD-472, or G3PD-967 assays was due to DNA degradation, not losses during extraction. However, as discussed above, much of the degradation occurred during the preparation of cornmeal or extraction of DNA from cornmeal.

Effect of Extrusion on DNA Degradation. Extrusion of maize grits and water was carried out at two barrel temperatures (60 and 170 °C max) and with torque settings ranging from 6 to 36 Nm. Results are presented for torque settings of 6, 18, and 36 Nm (**Figure 5**). The results for the other torque settings were intermediate between these settings (data not presented). Comparing the starting material (grits) with both the G3PD-68 and INCW-97 assays showed that the total amount of maize DNA extracted was approximately the same from all samples following extrusion at 60 °C. The G3PD-967 and INCW-980 assays showed that DNA extracted from untreated grits was also partially degraded, as was observed with the uncooked cornmeal samples (**Figure 4**). There was a clear effect of the torque settings when samples were extruded at 60 °C on detection of DNA capable of directing amplification of longer PCR products (e.g., G3PD-967 and INCW-980). Extrusion at 170 °C showed a much more marked effect on DNA degradation. No PCR amplification was detected for the products from assays G3PD-472, G3PD-967, INCW-581, or INCW-980. In contrast to the results at 60 °C, as torque increased above 6 Nm, the amount of small molecular weight DNA was markedly reduced (**Figure 5**). Virtually no DNA capable of directing amplification of the G3PD-68 and INCW-97 PCR products was extracted from samples extruded at 170 °C and a torque of 36 Nm.

Examination of DNA Fragment Length in Processed Foods. From a range of maize-containing processed food

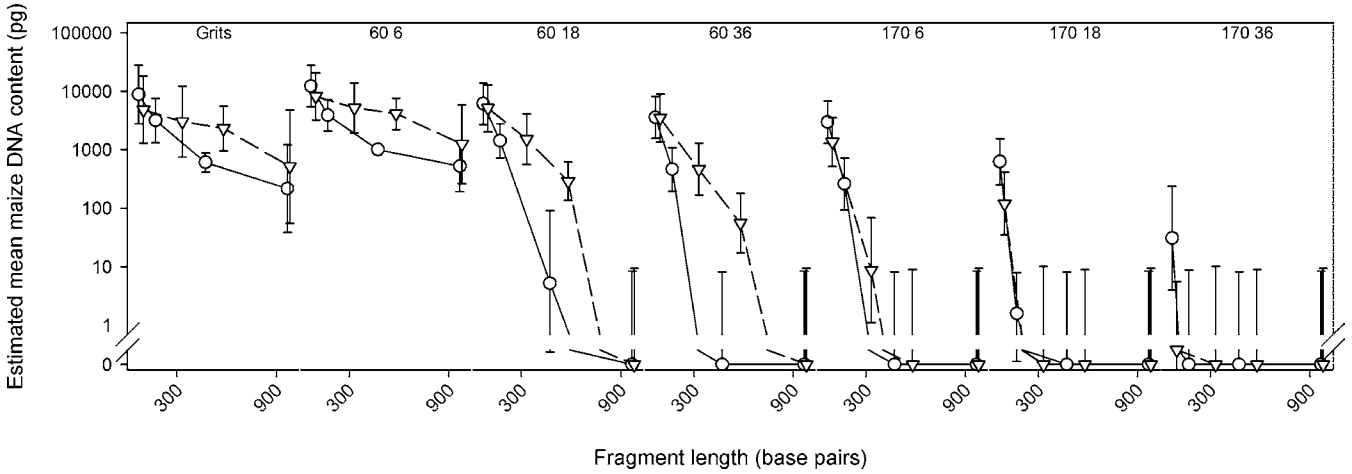

Figure 5. Estimation by real-time PCR of the effect of extrusion on mean maize DNA content for variable length target sequences using two temperatures and a range of torque values. The mean DNA content (in picograms) of aliquots of extractions representing 625 ng of maize grits or extruded starting material was estimated using variable length real-time PCR assays for the G3PD (triangle symbols) and INCW2 (circle symbols) sequences. Each point represents the estimated DNA amounts derived from four real-time PCR reactions. Error bars indicate 95% confidence intervals.

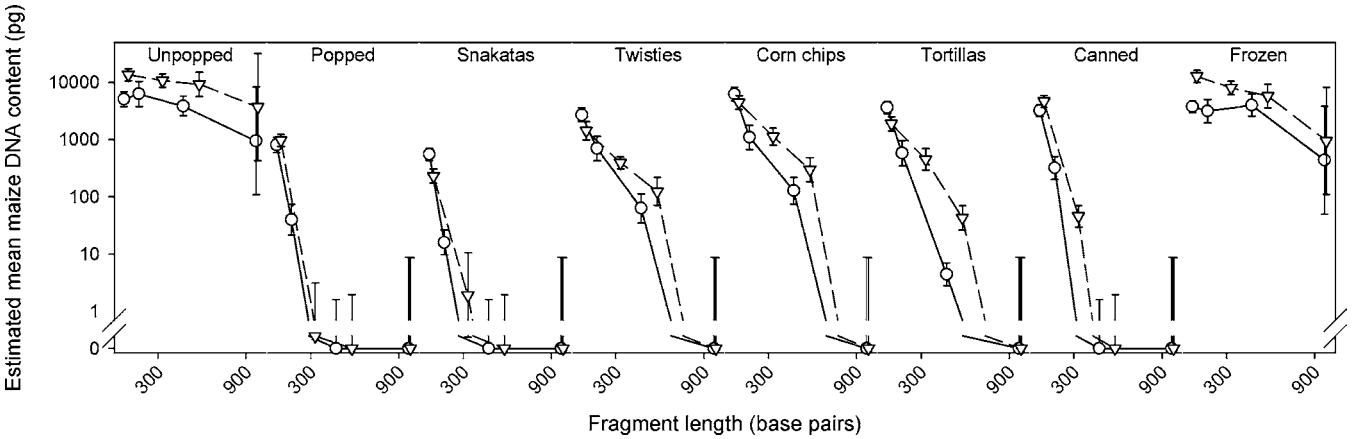

Figure 6. Real-time PCR estimation of mean maize DNA content for variable length target sequences in a range of maize-containing foodstuffs from supermarket shelves. The mean DNA content (in picograms) of aliquots of extractions representing 625 ng of starting material for dry food products or 1.25 mg of starting material for wet food products was estimated using the variable length real-time PCR assays for the G3PD (triangle symbols) and INCW2 (circle symbols) sequences. Each point represents the estimated DNA amounts derived from six real-time PCR reactions. Error bars indicate 95% confidence intervals.

products found on supermarket shelves, only the two maize food samples that had minimal processing, unpopped popping corn and frozen sweet corn, contained relatively intact DNA based on amplification of the G3PD-967 and INCW-980 PCR products (**Figure 6**). The ground unpopped corn behaved very similarly to the uncooked cornmeal samples in all assays (**Figures 4** and **6**). Popped corn DNA extractions contained much lower amounts of DNA (6.3- to 14-fold less than unpopped corn) even with the smallest, most sensitive assays, and no DNA was detectable with the largest assays. Other samples, including canned corn, tortillas, and corn-based snacks, showed either intermediate levels of DNA degradation or profiles of extreme degradation, similar to popped corn (**Figure 6**).

Comparison of G3PD versus INCW Assays. To compare the effect of sequence on the utility of this approach for estimating DNA degradation, the assays can be paired roughly on the basis of PCR product size, e.g., G3PD-68 vs INCW-97, G3PD-170 vs INCW-331, G3PD-472 vs INCW 581, and G3PD-972 vs INCW-980. Three of the G3PD-based assays amplify PCR products that are smaller than the corresponding INCW-based assays, however. On this basis, the G3PD-based assays might be expected to provide larger estimates of DNA content in processed samples than the INCW-based assays. Results presented in **Figures 4**−**6** suggest that, in general, assays based on the G3PD versus INCW sequences behave similarly for detecting DNA degradation, except that the INCW-331 and INCW-581 assays appear to consistently estimate that more maize DNA is available to amplify those target sequences than the corresponding G3PD-472 assay.

A direct comparison of the mean amount of DNA using the paired assays is presented in **Figure 7**. The relationship between the G3PD-68/INCW-97 and G3PD-170/INCW-331 pairs was as expected; i.e., G3PD assays generally estimated that more DNA suitable for amplification of PCR products was present after processing. Likewise, the G3PD-967/INCW-980 pair gave very similar estimates of DNA content, as expected from the very similar sizes of the PCR products. In contrast, the estimates from the G3PD-472/INCW-581 pair reverse the trend, and INCW-581 estimates slightly more DNA content than the G3PD-472 assay. Therefore, with the exception of the INCW-581 assay, these comparisons indicate that estimates of DNA content from the paired assays reflect the differences in PCR product length, and assays that amplify shorter PCR products estimate more maize DNA content.

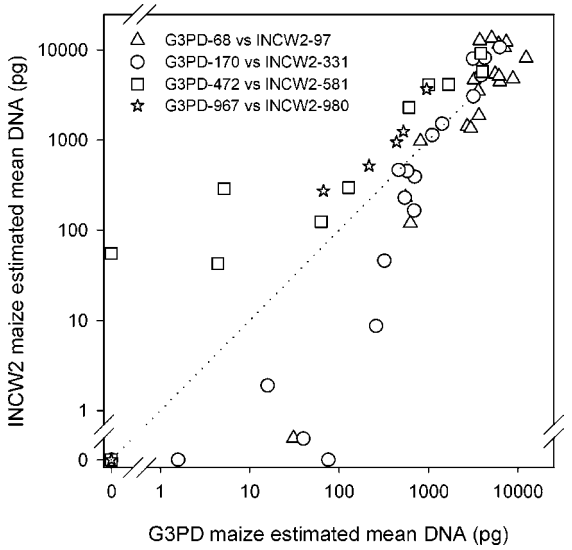

Figure 7. Comparison of the mean maize DNA content estimated by pairs of real-time PCR assays of variable length. The dotted line indicates where DNA content is identical for the two assays.

DISCUSSION

Assay Development. In this paper we describe a novel approach for measuring DNA degradation using sets of qPCR assays that amplify target sequences of varying length. The G3PD and INCW2 sequences were chosen for TaqMan assay development because they represent single copy or low copy sequences in the maize nuclear genome. Since G3P3 and INCW2 are endogenous maize genomic DNA sequences, the assays described in this paper are widely applicable for examination of DNA degradation or integrity in any maize-containing foods, regardless of diagnostic application (detection of GMO content derived from different transgenic events, ingredient contaminants, or microbial contaminants). INCW2 is a member of a small family of cell wall invertase genes. INCW2 maps to a single genomic location, on maize chromosome 2 (*27*). G3PD is the maize nuclear gene encoding the A subunit of chloroplastic glyceraldehyde-3-phosphate dehydrogenase. The maize genome also contains two related pseudogenes, pseudo(gpa1)1 (Genbank accession X15406) and pseudo-(gpa1)2 (*28*). The G3PD primer sets probably amplified DNA from three sequences, the G3PD gene and two pseudogenes. All primer and probe binding sequences are present in the pseudo(gpa1)1 sequence, although a number have a 1 bp mismatch with the published sequence (data not presented), including the probe sequence. The relative efficiency of detecting PCR products from the G3PD versus pseudo(gpa1)1 sequences using the G3PD-Pf probe sequence was not examined further. Probes with single base mismatches are used for discriminating different alleles in TaqMan genotyping applications (*29*). However, successful development of allelic discrimination assays requires that the Tm for probe binding and cycling conditions have been optimized for that application, as well as competition of two probes with and without the mismatch base. Our G3PD assays were not designed to meet these conditions. Therefore, we expect that the G3PD-Pf probe binds to the G3PD and pseudo(gpa1)1 sequences, permitting detection of these sequences. The lower C_t values obtained for all the G3PD assays versus the INCW2 assays (**Figure 2**) may be explained by the copy number differences between the two sequences.

PCR Analysis of DNA Degradation. DNA diagnostics based on PCR are becoming increasingly important for the food industry, particularly in the areas of detection of GMO-derived content, ingredient contamination, and microbial contamination. The processing of foods through various methods results in changes to the macromolecular constituents of the food, including denaturation or degradation of proteins and degradation of DNA. As less aggressive food processing techniques such as steaming and boiling are replaced by retorting, extrusion, popping, and canning, where temperatures may exceed 180 °C, the potential for degradation of DNA increases. Therefore, the effects of processing steps on DNA degradation must be understood for successful implementation of DNA diagnostics, particularly for detection of GMO-derived content and ingredient contamination.

When relying on PCR or real-time PCR, two considerations affect the ability to quantify DNA content and degradation in complex matrixes such as processed foods—differences in DNA recovery during extraction (either total recovery or fractionation based on size) and differences in sample inhibitor composition. Regarding DNA extraction, an assumption underlying this approach is equivalent efficiency of extraction of DNA from samples, regardless of DNA fragment length. Results using the shortest real-time PCR assays on cooked cornmeal (**Figure 4**) and on products extruded or cooked at low torque and temperature (**Figure 5**) confirm quantitative or near quantitative recovery of DNA in extractions; therefore, the reduced amplification of high molecular weight DNA targets using G3PD-967 and INCW-980 assays was most likely due to DNA degradation. For other samples where the amount of DNA recovered after extraction was low (popped corn, samples extruded at high torques and temperatures), it is less clear whether the results observed are due to DNA degradation or to nonquantitative extraction of DNA molecules from the food matrix. Inhibitors can also affect estimates of DNA content based on real-time PCR, due to reduction in PCR efficiency. However, similar effects might be expected on all assays, regardless of target amplicon length, resulting in the risk of overall underestimation of DNA content rather than overestimation of the extent of DNA degradation.

Many studies have looked at factors that influence the degradation of DNA in food during processing. For example, by comparing amplification of a small versus large PCR product, Hupfer et al. (*9*) showed that temperature and pH influenced degradation of a *cry*1A(b) transgene sequence in Bt maize during preparation of polenta. Chiter et al. (*10*) experimented with dry heating of maize kernels at a range of temperatures and confirmed that as little as 5 min at temperatures over 94 °C caused significant degradation of DNA, visible on an agarose gel by the absence of a high molecular weight band and by failure to amplify a 577 nucleotide PCR product from a maize gene. Bauer et al. (*11*) confirmed that the combination of pH and temperature affected degradation of isolated plasmid or plant genomic DNA and showed that processing of soy products into tofu or heated soymilk resulted in some DNA degradation so that PCR products of 1482 or 1719 bp did not amplify. Tilley (*12*) showed that the baking stage of bread making, in particular, resulted in degradation of wheat DNA, as determined by the ability of extracted DNA to contain templates for amplification of 900 bp versus 238 bp PCR products. Degradation of both transgenes and endogenous sequences during soy processing to soy powder, bean curd, and soymilk were examined by Chen et al. (*13*).

In the above papers, studies of DNA degradation in food used conventional PCR as the main analytical tool. At best, conventional PCR is semiquantitative, but usually is qualitative (band

present/band absent). Conventional PCR can identify processing steps that result in the complete loss of target molecules that are large enough to direct amplification of the PCR product being assayed (assuming the assay is optimized to be both sensitive and efficient), but does not estimate the concentration or number of DNA molecules present that are of a particular size.

Quantitative real-time PCR assays have been described for detecting GMO-derived content in foods, for example, containing maize (*17*, *30*), soy (*16*), and rape (*31*). In general, these assays have been developed to amplify short PCR products, so are likely to be suitable for sensitive detection of GMO-derived content in processed food samples containing degraded DNA. Commercially available GMO detection kits that employ qPCR using TaqMan technology, such as the "GMOQuant Maximizer Bt176 Corn" kit (GeneScan Europe, Bremen, Germany), typically amplify short PCR products.

Analysis of DNA Degradation—Relevance to Other Disciplines. Estimating the extent of DNA degradation as a result of natural or artificial processes is of interest to researchers in a variety of disciplines, for example, when analyzing the potential for horizontal gene transfer in the environment or human or animal gastrointestinal tracts involving transgene DNA from genetically modified organisms (*10*, *32*−*35*), forensic DNA diagnostics and studies of ancient DNA (*36*), and microbial cell viability and autolysis (*37*). Methods for examining DNA degradation included the use of conventional PCR assays of varying length (*10*, *34*), use of quantitative competitive PCR (QC-PCR; *35*, *38*), use of real-time quantitative PCR (*36*), analysis using HPLC (*37*), and examination of the ability of DNA to transform a target microorganism (*34*, *39*). Quantifying DNA degradation using qPCR assays of varying length, as described in this paper, may be of value to researchers in these disciplines.

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