

Research Article

Influence of Heat Processing on DNA Degradation and PCR-Based Detection of Wild-Type and Transgenic Maize

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Reliable detection of genetically modified (GM) maize is significant for food authenticity, labelling, quality, and safety assessment. This study aims to evaluate the factors influencing degradation and polymerase chain reaction (PCR) amplification of DNA from the wild type and transgenic maize (events Bt-176 and MON810) during thermal treatment at 100°C and 121°C. A new PCR method was developed targeting the *Cry1Ab* gene to detect insect-resistant GM plants. The yield of genomic DNAs extracted by the DNeasy plant mini kit dramatically decreased while DNAs obtained by cetyltrimethyl ammonium bromide- (CTAB-) based method did not show any visible changes in the yield by the time of processing. Treatment at 100°C did not significantly affect either genomic DNAs or amplicons. Heating at 121°C induced time-dependent degradation of genomic DNAs and exogenous *Cry1Ab* gene; however, it did not have any considerable influence on the exogenous 141 bp amplicons or endogenous amplicons in the range of 102 bp to 226 bp with the exception of the event MON810 extracted by the DNeasy plant mini kit. More yield was observed at 226 bp than 140 bp fragment of the invertase gene. The 141 bp fragment of the transgenic CaMV 35S promoter exhibited the highest thermal stability of all the examined amplicons. Analysis of foodstuffs demonstrated 102 bp amplicons specific for the *zein* gene as the effective marker to detect maize in the processed foods. The obtained results demonstrate that PCR-based detection of the wild type and transgenic maize is dependent on the combination of different parameters of crucial factors such as temperature and duration of exposure, transgenic event, DNA extraction method, DNA marker, and size and location of amplicons.

1. Introduction

In the recent period, widespread dissemination of transgenic plants worldwide has resulted in a dramatic spike of genetically modified organisms (GMOs) in food products [1]. At present, little is known about the risks of GM crops for human health and the environment. Hence, consumers have vague and varied opinions about GM products. To ensure consumers' freedom of choice, many countries have established regulatory systems and GMO labelling regulations. The European Union (EU) has introduced stringent legislative requirements for the labelling of food and feed containing GMOs or products derived thereof above a

threshold of 0.9% with a requirement for the traceability of the GMO in the food and feed chains [2, 3].

The efficient system for the monitoring and traceability needs reliable methods for detecting GMOs in raw materials and highly processed food. Numerous GMO detection methods have already been developed, validated, and harmonized worldwide [4, 5]. DNA diagnostics represent the most effective tool for the analysis of food ingredients as DNA is the most stable molecule during food processing. Moreover, the DNA-based polymerase chain reaction (PCR) is recognized as a reference method for GMO detection [6–8]. However, food production may cause fragmentation of genomic DNA and affect PCR analysis [9–20].

Correspondingly, accurate detection of GMOs in foodstuffs is a great challenge for modern food analysis. This demands comprehensive investigation of DNA stability and PCR-based GMO detection in processed foods.

Maize (*Zea mays* L.) is among the two major GM crops distributed worldwide. The cultivated area of transgenic maize represents about 32% of the global maize area [1]. Among GM plants, maize has the largest number (231) of transgenic events. Maize (corn) is one of the world's leading food crops. It is widely utilized as the raw material, supplement, and ingredient in food and feed production. In addition, maize belongs to important allergenic foods. Thus, accurate detection of the wild type and transgenic maize ingredients in processed foods is crucial for food authenticity, labelling, quality, and safety assessment.

At present, various PCR-based methods have been developed for maize identification and for the detection of GM varieties [5, 19–28]. The conventional PCR methods targeted at the *invertase* gene [23, 24], *Zea mays* 10 kDa *zein* gene [25], and quantitative real-time PCR assays targeted at *zein*, and *alcohol dehydrogenase I (Adh-1)* genes [26] and *starch synthase IIb gene* [27] were developed and successfully used for specific identification of corn. Different types of quantitative real-time PCR-based assays were applied for screening, identification, and quantification of transgenic events [26–29]. A number of studies have investigated the influence of food processing on plant DNA degradation and GMO detection [9–20]. The temperature, pressure, and pH were identified as important DNA degrading factors [10–15]. Hupfer et al. [10] described a conventional PCR method targeting the *CryIAb* gene for the detection of the insect-resistant maize in thermally treated products. The probability of detection was increased by choosing a short (211 bp) PCR amplicon. It has demonstrated the influence of pH during thermal treatment of maize on the detectability of the transgene [10]. Bauer and coauthors have revealed temperature and pH as important parameters influencing the degradation of plasmid and GM plant DNA from maize Bt176 and Roundup Ready soybean [11]. They monitored the degradation of plant DNA by determining the maximum detectable fragment length using conventional PCR and agarose gel electrophoresis.

Moreano and coauthors have shown that the accuracy of GMO quantification in thermally treated products is strongly affected due to disproportionate degradation rates of recombinant and reference targets when aiming at sequences of different lengths [16]. Moreover, ingredient particle size, degree and duration of technological treatment, and amplicon size negatively affect real-time PCR-based detection of food components [16]. Vijayakumar et al. [17] developed conventional PCR methods and adapted to target varying amplicon sizes of the trait, construct, and event-specific gene sequences in MON810 maize and Roundup Ready soybean. They identified the integrity of DNA, recovery, and PCR amplicon size as major factors for the successful detection of GMOs in processed foods. Godálová et al. [18] examined the combined effect of high temperature (121°C), elevated pressure (0.1 MPa), and low pH 2.25 on the quantification of MON810 maize. It was concluded that high

degree of processing leads to false-negative results of the transgenic constituent quantification if the difference in the size of the control and transgenic amplicons is 14 bp. Ballari and Martin [19] evaluated thermal and UV radiation treatment on the relative transgenic content of MON810 maize using plasmid DNA as a model system. Their study demonstrated that although food processing leads to substantial DNA degradation, it does not alter GMO quantification if the difference in the amplicon size between the transgenic and endogenous genes is as minimal as 16 bp. The discrepancies in the results of previous studies indicate that the effects of processing steps on DNA degradation must be clearly defined for each processed food on a case-to-case basis before any DNA-based method is applied to detect GMOs [17]. Therefore, it is a pressing task to accumulate basic data regarding the impact of food processing on the DNA integrity and PCR-based detection.

The given study aims at evaluating the effect of thermal treatment on the DNA integrity and amplification of the wild type and transgenic maize. Various parameter combinations of the factors influencing DNA degradation and PCR amplification, such as temperature, duration of treatment, DNA extraction method, wild-type maize and GM events, amplicon size, endogenous and transgenic amplicons, and PCR primer pairs are investigated for the first time. A new PCR method targeting the transgenic *CryIAb* gene is presented for reliable screening of Bt plants.

2. Materials and Methods

2.1. GMO and Non-GMO Materials. Certified reference materials, such as maize GMO Standard ERM-BF-411 containing 1% Bt-176 and maize GMO Standard ERM-BF-413 containing 1% MON810, were purchased commercially (Sigma-Aldrich). The seeds of maize (*Zea mays* var. *indentata*) and foodstuffs were obtained from local markets in Tbilisi (Georgia). The products of both local and foreign provenance were utilized. In particular, maize seeds (Lomtagora), maize flour 1 (G group), maize flour 2 (Laiti), cornflakes 1 (Khramuna), cornflakes 2 (Burbu), and popcorn (G&V) are manufactured in Georgia, while the other ones are imported, such as marinade—sweet corn (Coo-poliva, Spain), cereal granules—Start cocoa balls grain breakfast cereals (Lantmannen Axa, Ukraine), and baby food—Nestlé dairy-free multicereal porridge 5 cereals (Nestle Russia, Russia). The maize seeds were ground by electric grinder (Siemens, Munich, Germany) to obtain flour. The certified reference materials, such as GM maize powder as well as baby food and two kinds of maize flour, were used directly for DNA extraction. However, foodstuffs such as marinade, cornflakes, cereal granules, and popcorn were frozen in liquid nitrogen and ground using a mortar and pestle.

2.2. Sample Preparation: Thermal Processing. In the given research, dry heating at 100°C and 121°C was applied to treat products. To evaluate various parameter combinations of several factors under different processing conditions, four

independent experiments were conducted. To study the impact of genetic modification, the powders of certified reference materials of two GM maize varieties, namely, Bt-176 and MON810, as well as flour of the wild-type (non-transgenic) maize were investigated. In particular, GM maize event Bt-176 was applied in the first, second, and third experiments, while GM maize event MON810 was used in the fourth experiment. In addition, nontransgenic maize was analysed in each experiment. 50 mg of samples were placed separately in each of the four 1.5 ml Eppendorf plastic tubes. To assess the effect of heat and treatment duration, the first tube was put at room temperature (20°C) for 300 min as a control (untreated) sample, while three tubes were exposed to heating during 60 min, 180 min, and 300 min, respectively, in AccuBlock Digital Dry Baths (Labnet International, Edison, NJ, USA). The samples were treated at 100°C in the first experiment, while treatment at 121°C was applied in other three experiments.

2.3. Genomic DNA Extraction. To evaluate the impact of the DNA extraction method, genomic DNA was extracted from 50 mg of sample by two methods. In particular, cetyltrimethyl ammonium bromide- (CTAB-) based method [30] was used in the first and second experiments, whereas the DNeasy plant mini kit (Qiagen, Hilden, Germany) was applied in the third and fourth experiments. The genomic DNAs of foodstuffs were extracted by the CTAB-based method. The purity and concentration of extracted DNAs were estimated by using spectrophotometer DeNovix DS-11 (DeNovix Inc. Wilmington, USA). The quantity and integrity of DNA were evaluated using electrophoresis (VWR International, Radnor, Pennsylvania, USA) on 1% agarose gel (SeaKem LE agarose; Cambrex, East Rutherford, New Jersey, USA) containing 1 µg/ml of ethidium bromide (Sigma-Aldrich, St. Louis, Missouri, USA). The agarose gels were visualized under ultraviolet (UV) light, and a digital image was obtained using a gel documentation system PhotoDoc-It imaging system (UVP, Upland, California, USA).

2.4. PCR Analysis. The PCR primers used in this study are shown in Table 1. We designed new GMO-specific primer pair, namely, Cry124f/Cry124f-targeted *Cry1Ab* delta-endotoxin (*Cry1Ab*) gene (GenBank acc no. AY326434.1) using bioinformatics tools and software, such as Primer-Quest tool (Integrated DNA Technologies, Coralville, Iowa, USA), sequence alignment tool Align_MTX [31], and FastPCR [32]. The forward primer corresponds to the sequences between 2028 and 2050 nt of the *Cry1Ab* gene. The reverse primer is complementary to the sequences between 2151 and 2130 nt of the *Cry1Ab* gene. The other PCR primers were taken from our previous studies and other publications [22–25]. The synthesis of oligonucleotide primers was performed by Integrated DNA Technologies (IDT).

Amplification reactions were carried out using Standard Taq Buffer, 1.25 units of *Taq* DNA polymerase, 1.5 mmol of MgCl₂, and 0.2 mMol of each dNTP (deoxynucleotide solution mix) from New England BioLabs (Ipswich, Massachusetts,

USA), 0.5 µM of each primer, and 50 ng of genomic DNA in a final volume of 25 µl.

PCRs were performed in a Techne TC-412 thermal cycler (Techne, Minneapolis, Minnesota, USA) with the amplification conditions suitable for each primer pair (Table 2).

The amplified products were determined by electrophoresis on 2.0% agarose gels; afterwards, they were evaluated under ultraviolet (UV) light and photographed using a gel documentation system PhotoDoc-It imaging system.

2.5. Statistical Analysis. The amounts of genomic DNAs measured spectrophotometrically were analysed by the program SigmaPlot 12.5 (Systat Software Inc., San Jose, California, USA). The band intensity of PCR products on gels was determined by the image processing program ImageJ (version 1.51q, Institute Pasteur, Paris, France). This software allowed semiquantitative measurement of PCR amplicons by digital image analysis of electrophoretic gel, as was previously reported [15, 33]. The data obtained from different PCR experiments were compared with each other by one-way analysis of variance (ANOVA). The analyses were performed with GraphPad Prism 6 software for Windows (GraphPad Software). The results are expressed as mean values plus standard deviation at a set significance level of $P < 0.05$.

3. Results and Discussion

Two widely distributed transgenic events of maize, namely, insect-resistant Bt corn varieties Bt-176 and MON810 were examined. They are approved as food and feed in several countries including the European Union (EU); moreover, MON810 is the only transgenic cultivar grown in European countries. In each experiment, DNA of the GM maize event was compared with the DNA of unmodified maize. The analytical procedure included several sequential steps: sample preparation (1); thermal treatment (2); genomic DNA extraction (3); DNA assessment by agarose gel electrophoresis and spectrophotometer (4); PCR amplification of endogenous amplicons (5); PCR amplification of GMO-specific amplicons (6); comparison and interpretation of results (7). In this study, we have investigated the effect of different combinations of several important factors on the GMO detection. These factors included temperature and duration of heat processing, DNA extraction tools, non-transgenic and transgenic maize events, endogenous and exogenous DNA markers, PCR primers, and size and location of amplicons. The following combinations were applied: experiment 1: wild type and event Bt176 maize treated at 100°C and extracted by the CTAB method; experiment 2: wild type and event Bt176 maize treated at 121°C and extracted by the CTAB method; experiment 3: wild type and event Bt176 maize treated at 121°C and extracted by the DNeasy plant kit; and experiment 4: wild type and event MON810 maize treated at 121°C and extracted by the DNeasy plant kit. These GM crops and various factors have not yet been investigated before under the abovementioned processing conditions.

TABLE 1: Oligonucleotide primers.

Target	Primers	Sequence	Ampacon (bp)	Reference
Maize zein gene	ZEINI/ZEINr	ACACCACCGACCATGGCAGC TGGTGGCAAGTGCCTGGAA	102	[25]
Maize invertase gene	IVRI/IVRr	TCTCCCGTGATCCTGCCCCG GCTTGGACCGTGCCTTCCT	140	[23]
Maize invertase gene	IVTASI/IVTAS2	CCGCTGTATCACAAGGGCTGGTACC GGAGCCCGTGTAGAGCATGACGATC	226	[24]
CryIAb gene	CryIf/CryI r	GCACCTCCGTGGTGAAGGGC AACCCACGGTGCCTGAAGCTG	258	[22]
CaMV 35S promoter	P3581/P35Sr	CGTGCACCATGATGTGTGATTTCGAC GIGGGATMTGCGTCATCCCTT	141	[22]
CryIAb gene	Cry124I/ Cry1 24f	CATCCTCAACAGCATCACTATCT GCCGTAGAGAGGAAAGGTTAAAC	124	This study

TABLE 2: Conditions of PCR.

Primers	Cycles	Initial denaturation		Denaturation		Annealing		Elongation		Final extension	
		Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)	Time (min)
IVTAS, IVR, ZEIN	40	95	3	95	0.30	63	0.30	72	0.35	72	5
P35S	40	95	3	95	0.30	65	0.30	72	0.35	72	5
CryI	50	95	3	95	0.25	62	0.30	72	0.45	72	7
Cry124	50	95	3	95	0.25	62	0.30	72	0.35	72	5

3.1. Impact of Heat on Genomic DNA. We applied 100°C and 121°C for thermal treatment as frequently adopted temperatures in food production and preservation. In particular, pasteurization of certain products is performed at 100°C, while sterilization is carried out at 121°C. The electrophoretic images of genomic DNAs (Figure 1) have revealed that the impact of thermal treatment largely depends on the temperature and duration of exposure.

A significant difference was found between samples processed at different temperatures. The influence of 100°C on DNA integrity was significantly weaker than that of 121°C. The high-molecular-weight genomic DNA bands of high intensity appeared in all untreated samples. The large amounts of the entire genomic DNAs with weak smearing were maintained in the flour treated at 100°C (Figure 1(a)). However, DNA extracts from the samples processed at 121°C contained strong smeared DNA bands, while the size of DNA fragments was reduced by the time of processing (Figures 1(b)–1(d)). Therefore, the obtained results clearly indicate that high temperature affected DNA integrity. Moreover, exposure at 121°C caused more severe degradation than heating at 100°C. In addition, a more significant impact was observed in samples obtained by the DNeasy kit (Figures 1(c) and 1(d)) than those obtained by means of the CTAB method (Figures 1(a) and 1(b)) due to their different approach to DNA purification. No important differences were found between GM events or between transgenic and nontransgenic varieties.

Spectrophotometric analysis demonstrated significant influence of the DNA extraction method on the yield of genomic DNA (Figure 2). In particular, the CTAB method gave higher yield of DNA than the DNeasy kit. Especially

significant difference was observed in case of the highly processed samples. Thermal treatment at 121°C for 300 min did not have any impact on the quantity of DNA extracted by the CTAB method in case of both wild type and GM maize (event Bt176). However, heat processing at 121°C had a severe effect on the quantity of DNA extracted by the DNeasy kit (Figure 2). Moreover, the DNA yield for each of wild type and GM events (Bt176 and MON810) dramatically decreased with the increase in the time of exposure. In particular, DNA yield decreased by 50–60% after 60 min and by 90% after 300 min compared with the untreated samples. This may be explained by different approaches to DNA purification; namely, CTAB extracts consist of all DNA fragments generated by processing, while short DNA fragments generated after thermal degradation are removed from the DNA extracts produced by the DNeasy kit. Thus, the CTAB method is more suitable than the DNeasy kit for DNA extraction from processed foodstuffs. The results obtained indicate that temperature and duration of thermal processing, as well as the DNA extraction method, are crucial factors influencing DNA integrity and quantity. These outcomes are in accordance with the previous findings [12–15, 30].

3.2. Impact of Heat on the Endogenous Amplicons. In order to evaluate the extension and degree of DNA degradation, we investigated amplicons of different lengths and various genetic locations. GMO quantitative analysis implies reliable detection of both endogenous and transgenic targets. Correspondingly, comparative analysis of their degradation during food processing is significant for the accurate

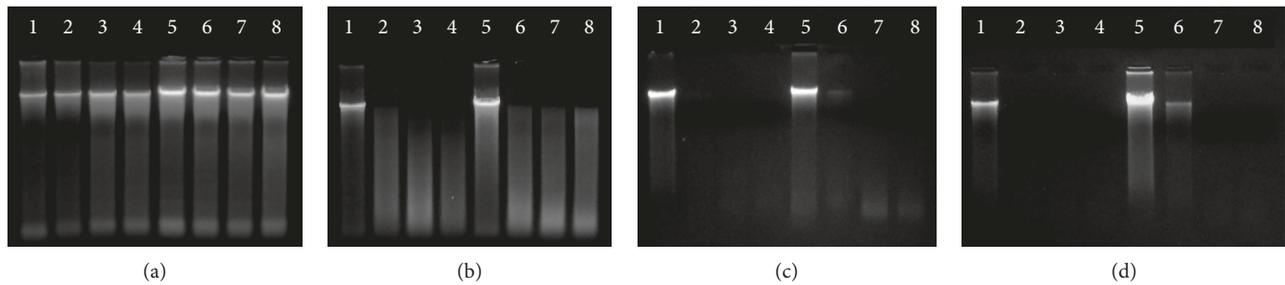


FIGURE 1: Genomic DNAs from the samples treated at high temperature: (a) 100°C and (b–d) 121°C. Exposure duration: lanes 1 and 5 = 0 min; lanes 2 and 6 = 60 min; lanes 3 and 7 = 180 min; lanes 4 and 8 = 300 min. Extraction was performed by the CTAB method (a, b) and DNeasy kit (c, d). Samples: wild-type maize (lanes 1–4 in (a–d)), event Bt176 (lanes 5–8 in (a–c)), and event MON810 (lanes 5–8 in (d)).

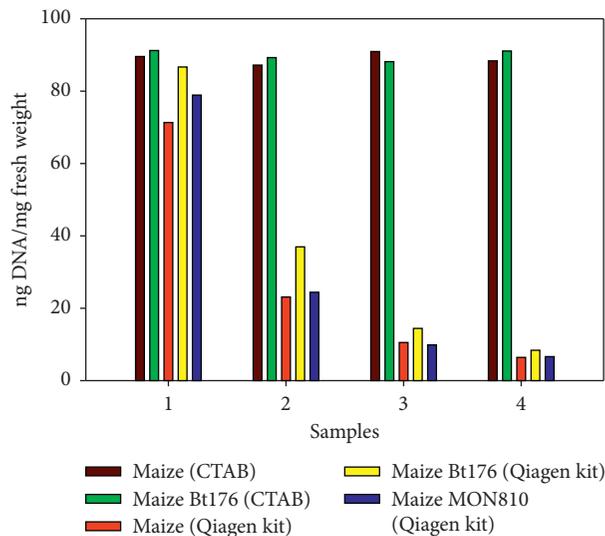


FIGURE 2: Spectrophotometric analysis of DNA extracted from samples: wild-type maize by the CTAB method = maize (CTAB) and DNeasy kit = maize (Qiagen kit), event Bt176 by the CTAB method = maize Bt176 (CTAB) and DNeasy kit = maize Bt176 (Qiagen kit), and event MON810 by the DNeasy kit = maize MON810 (Qiagen kit) treated at 121°C. Exposure duration: 1 = 0 min; 2 = 60 min; 3 = 180 min; 4 = 300 min.

detection of GM foods [34–36]. Due to this evidence, we examined maize-specific and GMO-specific amplicons. The PCR products were selected within the size range between 102 bp and 258 bp, foreseeing the efficiency of short amplicons for the analysis of foodstuffs [6, 22]. The amplicons were produced by conventional end-point PCR as it is a rapid and cheap technique for DNA amplification. Moreover, it was previously successfully used in similar studies [10, 11, 17]. However, comparison of PCR-electrophoresis gels enables semiquantitative evaluation, while the real-time PCR allows precise quantitative analysis.

Three maize-specific amplicons ranging between 102 bp and 226 bp were applied to evaluate DNA degradation and PCR-based detection of maize. These amplicons included the 102 bp fragment of the zein gene as well as 140 bp and 226 bp fragments of the invertase gene. As shown in Figure 3, agarose gel electrophoresis of PCR products demonstrated one amplicon of expected length in each PCR reaction for all maize samples (Figure 3, lanes 1–8). In particular, the oligonucleotide primers IVTAS1 and IVTAS2 produced 226 bp fragment (Figures 3(a)–3(d)), while primer pairs IVRf/IVRr

(Figures 3(e)–3(h)) and ZEINf/ZEINr (Figures 3(i)–3(l)) generated 140 bp and 102 bp products, respectively. It should be emphasized that almost no degradation was seen for these amplicons from all of the samples of wild-type maize and event Bt176 treated at both 100°C and 121°C and extracted by both the CTAB method and Qiagen kit (Figure 3). However, maize specific amplicons of MON810 heated at 121°C and obtained by the Qiagen kit showed significant degradation by the time of exposure (Figures 3(d), 3(h), and 3(l)), indicating the importance of the transgenic event in PCR results. A higher yield of 226 bp than 140 bp fragment of the invertase gene suggests the significance of PCR primers. Thus, our study has demonstrated that the transgenic event and selection of primers play an important role in the accuracy of PCR-based analysis of foods. The absence of any amplification product in all water negative controls (Figure 3, lane 9) has indicated the absence of contaminating DNA from the environment, buffers, or reagents. The obtained results have revealed that PCR methods applied in this study may be used for maize detection in thermally processed products, as it was previously reported [23–25].

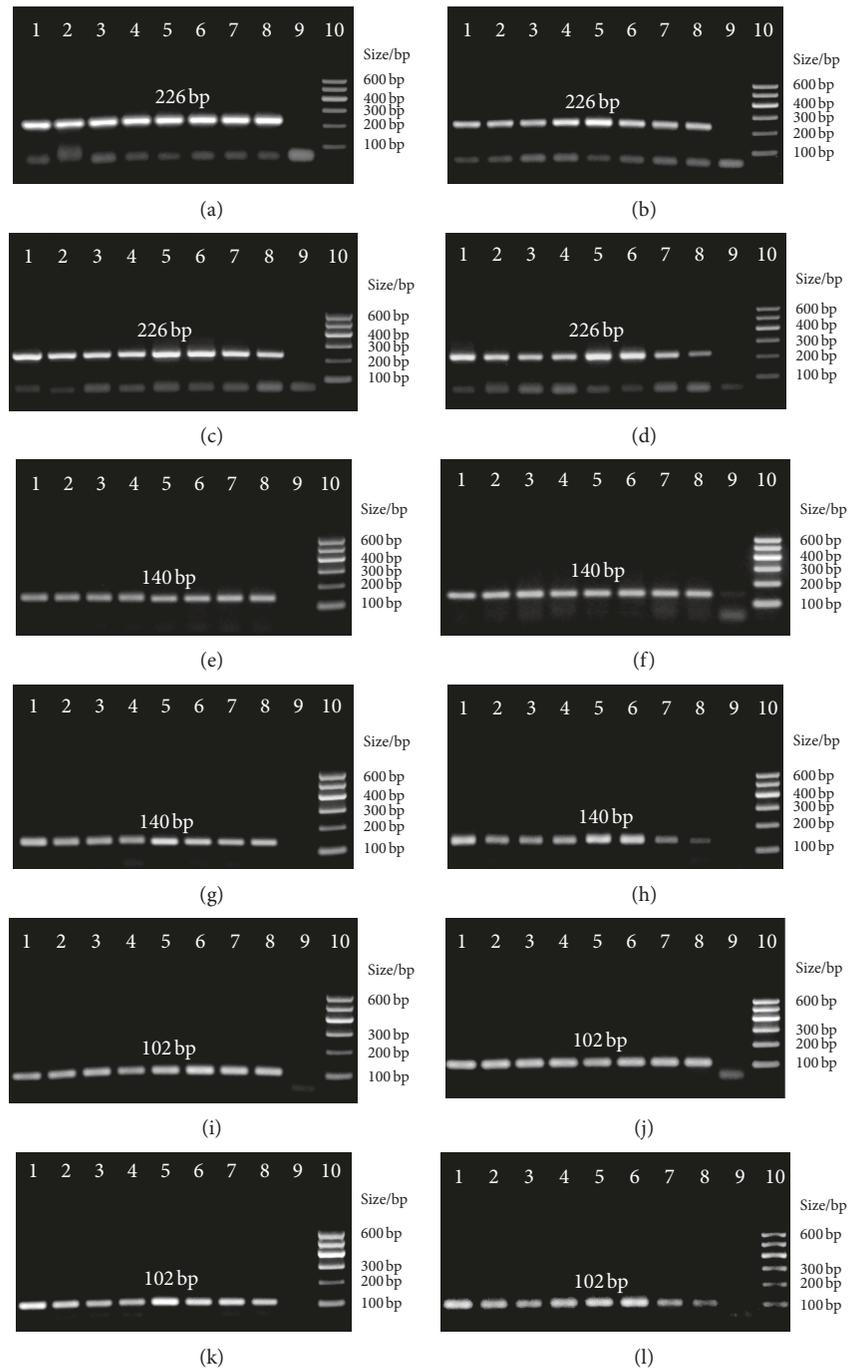


FIGURE 3: PCR analysis of endogenous amplicons with size of (a–d) 226 bp, (e–h) 140 bp, and (i–l) 102 bp from the samples treated at high temperature: 100°C (a, e, i) and 121°C (b–d, f–h, j–l). Exposure duration: lanes 1 and 5 = 0 min; lanes 2 and 6 = 60 min; lanes 3 and 7 = 180 min; lanes 4 and 8 = 300 min. Extraction was performed by the CTAB method (a–b, e–f, i–j) and DNeasy kit (c, d, g, h, k, l). Samples: wild-type maize (lanes 1–4 in (a–l)), event Bt176 (lanes 5–8 in (a–c, e–g, i–k)), event MON810 (lanes 5–8 in (d, h, l)), water (lane 9 in (a–l)), and GelPilot 100 bp ladder (Qiagen) (lane 10 in (a–d)).

3.3. Impact of Heat on the GMO-Specific Amplicons. In order to compare the degradation of the endogenous and transgenic DNA sequences, both maize-specific and GMO-specific amplicons of similar sizes were selected. We investigated three abovementioned maize-specific amplicons versus GMO-specific amplicons, namely, 141 bp amplicon corresponding to cauliflower mosaic virus (CaMV) 35S promoter

and two amplicons of 258 bp and 124 bp corresponding to the transgenic *Cry1Ab* gene. The CaMV 35S promoter and *Cry1Ab* gene is present in both GM maize events Bt176 and MON810 examined in this paper. The transgenic regions of GM plants often contain the CaMV 35S promoter to regulate the transcription of the inserted genes [7]. The *Cry1Ab* gene of insecticidal protein *Cry1Ab* δ endotoxin from *Bacillus*

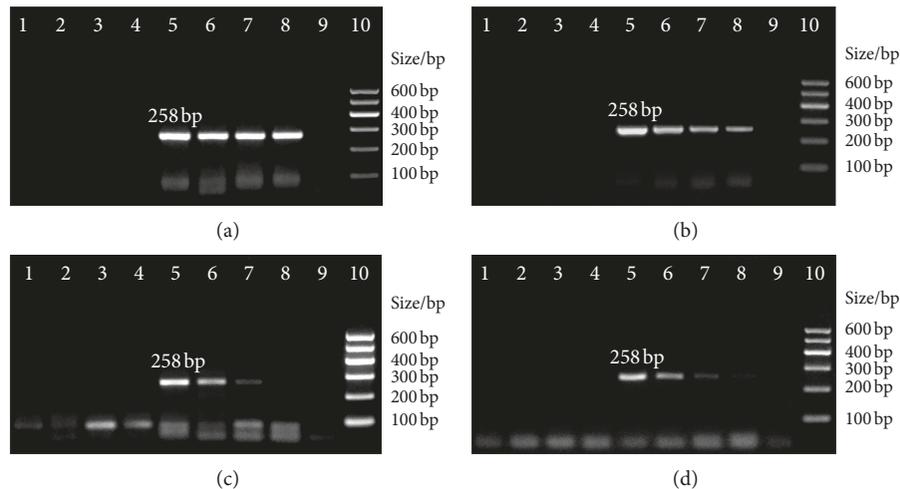


FIGURE 4: PCR analysis of exogenous 258 bp amplicon from the samples treated at high temperature: (a) 100°C and (b–d) 121°C. Exposure duration: lanes 1 and 5 = 0 min; lanes 2 and 6 = 60 min; lanes 3 and 7 = 180 min; lanes 4 and 8 = 300 min. Extraction was performed by the CTAB method (a, b) and DNeasy kit (c, d). Samples: wild-type maize (lanes 1–4 in (a–d)), event Bt176 (lanes 5–8 in (a–c)), event MON810 (lanes 5–8 in (d)), water (lane 9 in (a–d)), and GelPilot 100 bp ladder (Qiagen) (lane 10 in (a–d)).

thuringiensis ssp. *Kurstaki* is inserted in the transgenic Bt crops to give them insect resistance trait. The primer pair P35Sf/P35Sr generating the 141 bp product and primer pair Cry1f/Cry1r generating the 258 bp product was taken from our previous study [22]. In this study, new oligonucleotide primers Cry124f and Cry124e were designed, and PCR method was developed for the detection of 124 bp fragment of the transgenic *Cry1Ab* gene. This method was successfully optimized and validated using certified reference materials of Bt176 and MON810.

Figure 4 shows amplification results for the 258 bp fragment of the transgenic *Cry1Ab* gene. The PCR product of appropriate size was seen in GM maize samples (Figure 4, lanes 5–8), while nontransgenic samples (Figure 4, lanes 1–4) did not produce any PCR fragments. This confirms the high specificity of the previously described method [22]. No amplification products were seen in the water samples (Figure 4, lane 9). This indicated the absence of contamination. Agarose gel electrophoresis of PCR products demonstrated that heating at 100°C for 300 min had no influence on the production of the 258 bp fragment (Figure 4(a), lanes 5–8). However, treatment at 121°C induced a time-dependent reduction of the amplicon yield (Figures 4(b)–4(d), lanes 5–8) that affects the accuracy of GMO detection. In addition, PCR products were not detected for either Bt176 or MON810 DNA samples extracted by the Qiagen kit after exposure at 121°C for 300 min (Figures 4(c) and 4(d), lane 8). These experiments showed that the CTAB method yielded more amplifiable DNA from processed samples as compared to the Qiagen kit. This outcome is in accordance with the result mentioned above for the genomic DNA; namely, the CTAB method gave higher yield of DNA than the DNeasy kit (Figures 1 and 2). In this case, no important difference was seen between transgenic varieties, such as Bt176 and MON810. These outcomes have revealed the important role of temperature, duration of exposure, and DNA extraction

method in the PCR-based GMO detection. Our results coincide with the findings observed previously [12–18].

The PCR analysis of the short transgenic amplicons, namely, 141 bp fragment of the CaMV 35S promoter and 124 bp fragment of the *Cry1Ab* gene, revealed one expected product for GM maize samples, while no PCR product was seen for nontransgenic samples (Figures 5(a)–5(d), lanes 1–4). This shows the high specificity of these PCR methods. In addition, gel electrophoresis revealed the absence of contamination in water negative controls (Figures 5(a)–5(d), lane 9). The treatment at 121°C for 300 min did not have any impact on the amplification of the 141 bp amplicon (Figures 5(a) and 5(b)). However, the intensity of the 124 bp amplicon decreased by the time of exposure, for both GM maize events, such as Bt176 and MON810 (Figures 5(c) and 5(d)) that correspond with the time-dependent degradation of the 258 bp amplicon of the same gene (Figure 4). Therefore, it has revealed an unexpected result of higher thermostability of 141 bp than that of 124 bp amplicon (Figure 5). This indicates the importance of amplicon stability.

In addition, comparison of endogenous and transgenic amplicons exhibited size-dependent degradation with the exception of the 141 bp transgenic fragment which was more stable than the 140 bp fragment of the invertase gene and 102 bp fragment of the zein gene in the event MON810. We assume that a higher stability of the 141 bp amplicon in comparison with other short amplicons may be due to their different locations in the genome. This proves the significant role of the DNA marker in the GMO detection. Our findings coincide with the results of Martin-Fernaandez et al. [37]. They have reported that the sensitivity of the detection of wheat was considerably affected by the marker gene. Comparison of GM events has revealed similar thermostability of 141 bp and 258 bp fragments, while 124 bp amplicon degraded faster in MON810 than in Bt176.

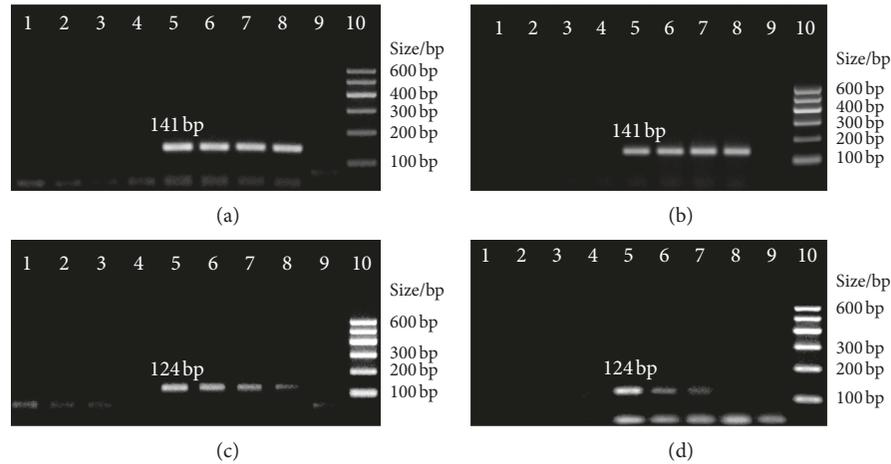


FIGURE 5: PCR analysis of exogenous amplicons with size (a-b) 141 bp and (c-d) 124 bp from the samples treated at 121°C. Exposure duration: lanes 1 and 5 = 0 min; lanes 2 and 6 = 60 min; lanes 3 and 7 = 180 min; lanes 4 and 8 = 300 min. Extraction was by the DNeasy kit. Samples: wild-type maize (lanes 1–4 in (a–d)), event Bt176 (lanes 5–8 in (a, c)), event MON810 (lanes 5–8 in (b, d)), water (lane 9 in (a–d)), and GelPilot 100 bp ladder (Qiagen) (lane 10 in (a–d)).

Thus, it is evident that transgenic events, size and location of the amplicons, and the choice of primers play an important role in the reliable detection of GMOs in processed foods. The study of different parameter combinations of several important factors suggests that food analysis should be performed for each separate case, taking into account each parameter influencing PCR-based detection of GM food.

In addition, four independent PCR experiments were carried out for each PCR amplicon in order to implement a statistical analysis. PCR products for each amplicon were run side by side on the same agarose gel electrophoresis (Figure 6(a)). The data obtained from different PCR experiments were compared with each other. Results are expressed as mean values plus standard deviation (Figure 6(b)).

3.4. PCR Analysis of Food Products. In order to investigate the usefulness of the abovementioned PCR methods, different types of foodstuffs were analysed. The maize flours 1 and 2 consist only of the maize material and undergoes mechanical treatment. The marinade, cornflakes, and popcorns consist of maize as a major ingredient; however, they undergo both mechanical and thermal treatments. The cereal granules and baby food contain a low percentage (1.7% and 6.3%) of maize ingredients. In addition, baby food undergoes mechanical treatment, while the cereal granules were produced by mechanical and thermal treatments.

The degradation of both endogenous and exogenous amplicons was studied by PCR analysis of different types of processed foods using appropriate primers (Figure 7). PCR amplification of both 226 bp and 140 bp fragments of the maize invertase gene produced one amplicon of expected size in all samples with the exception of cereal granules and popcorn (Figures 7(a) and 7(b), lanes 6–7). However, 102 bp PCR amplicon-targeted maize zein gene was generated in all foodstuffs (Figure 7(c)). No amplification product was seen in water samples that indicated the absence of contamination (Figures 7(a)–7(f), lane 10).

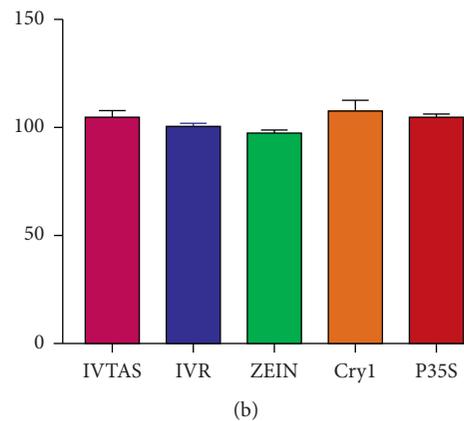
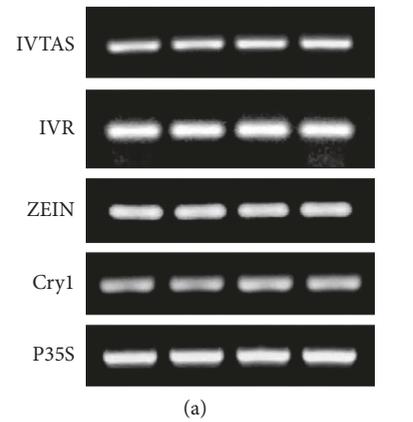


FIGURE 6: Results of statistical analysis: (a) agarose gels of amplicons from four independent experiments; (b) results are expressed as mean values plus standard deviation at a set significance level of $P < 0.05$. Amplicons: IVTAS = 226 bp, IVR = 140 bp, ZEIN = 102 bp, Cry1 = 258 bp, and P35S = 141 bp.

The results have revealed that the studied food products have different levels of maize ingredients. The detection of maize is dependent on three factors, such as the content of the maize ingredient in the foodstuff, processing level of the

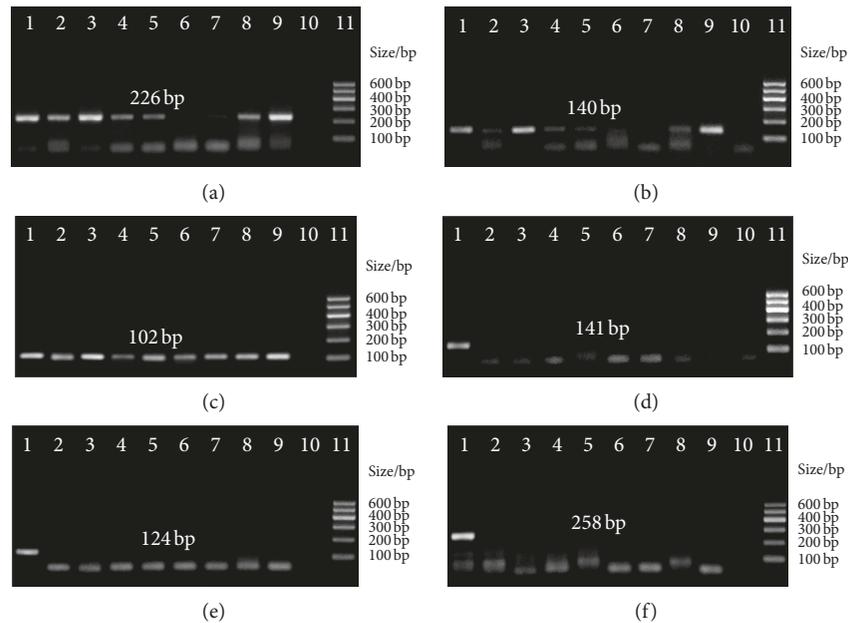


FIGURE 7: PCR analysis of food products (a–f): lane 1 = event MON810 (1%); 2 = marinade; 3 = maize flour 1; 4 = cornflakes 1; 5 = cornflakes 2; 6 = cereal granules; 7 = popcorn; 8 = baby food; 9 = maize flour 2; 10 = water; 11 = GelPilot 100 bp ladder (Qiagen). Endogenous amplicons with size (a) 226 bp, (b) 140 bp, and (c) 102 bp. Exogenous amplicons with size (d) 141 bp, (e) 124 bp, and (f) 258 bp.

product, and DNA marker. The GM MON810 maize powder, maize fours 1 and 2, exhibited the highest intensities of amplified products, as was expected. The marinade, cornflakes, and baby food showed PCR bands of similar intensities. The cereal granules and popcorn undergo more severe processing than other foodstuffs. This severe processing caused the degradation of 226 bp and 140 bp amplicons in cereal granules and popcorn. The outcomes demonstrate that the selection of an appropriate DNA marker is extremely important for reliable food analysis. Moreover, 102 bp amplicon was identified as the effective marker for maize detection in processed foods. PCR analysis with GMO-specific primers did not give any positive results. This indicates that these foodstuffs do not contain GM ingredients (Figures 7(d)–7(f)).

4. Conclusions

In the given research, DNA degradation and PCR amplification of transgenic and nontransgenic maize DNA were investigated under thermal treatment at 100°C and 121°C for 300 min. The influence of different factors has been monitored, namely, temperature, duration of treatment, DNA extraction methods, size and location of amplicons, DNA markers, PCR primers, and GM events. A new PCR-based method targeted at the transgenic *CryIAb* gene has been worked out for the detection of insect resistant GM crops. The obtained results clearly indicate that heating at 100°C has a slight impact on the DNA integrity and PCR amplification. However, 121°C has induced time-dependent degradation of the genomic DNA, and it has considerable influence on the PCR amplification of certain amplicons and may affect GMO detection. The CTAB method has given a higher yield of genomic and amplifiable DNA than the Qiagen kit, particularly from processed samples. Investigation of endogenous

amplicons has proved that short fragments between 102 bp and 226 bp were stable with the exception of samples from transgenic event MON810. The fragments of the exogenous *CryIAb* gene have revealed similar degradation for both GMO events. The transgenic 141 bp amplicon targeted at the CaMV 35S promoter showed the highest thermostability of all tested amplicons and was identified as an efficient marker for the analysis of highly processed GM foods.

Therefore, the outcomes of this study clearly demonstrate that various processing parameters, such as temperature, duration of exposure, and DNA extraction method, as well as transgenic event, DNA markers, and size and location of the amplicons, are critical factors influencing PCR-based identification of food ingredients and the reliability of GMO detection. The PCR methods applied in the given research show high specificity and sensitivity for the reliable detection of maize and GMOs in processed products.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

- [1] C. James, *Global Status of Commercialized Biotech/GM Crops: 2017*. ISAAA Brief No. 53-2017, ISAAA (*The International Service for the Acquisition of Agri-Biotech Applications*), Cornell University, Ithaca, NY, USA, 2017, <http://www.isaaa.org>.
- [2] European Commission, "Regulation (EC) no. 1829/2003 of the European parliament and of the council of 22 september 2003 on genetically modified food and feed," *Official Journal of the European Union*, vol. 268, pp. 1–23, 2003.
- [3] European Commission, "Regulation (EC) no. 1830/2003 of the European parliament and of the council of september 2003 concerning the traceability and labeling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending directive 2001/18/EC," *Official Journal of the European Union*, vol. 268, pp. 24–28, 2003.
- [4] W. Dong, L. Yang, K. Shen et al., "GMDD: a database of GMO detection methods," *BMC Bioinformatics*, vol. 9, no. 1, p. 260, 2008.
- [5] *Compendium of Reference Methods for GMO Analysis*, European Union Reference Laboratory for GM food and feed (EURL-GMFF), European Network of GMO Laboratories (ENGL), Publication Office of the European Union, Luxembourg, UK, 2011.
- [6] C. M. Alarcon, G. Shan, D. T. Layton, T. A. Bell, S. Whipkey, and R. D. Shillito, "Application of DNA- and protein-based detection methods in agricultural biotechnology," *Journal of Agricultural and Food Chemistry*, vol. 67, no. 4, pp. 1019–1028, 2019.
- [7] G. Randhawa, M. Singh, and P. Sood, "DNA-based methods for detection of genetically modified events in food and supply chain," *Current Science*, vol. 110, no. 6, pp. 1000–1009, 2016.
- [8] G. Cottenet, C. Blancpain, V. Sonnard, and P. F. Chuah, "Two FAST multiplex real-time PCR reactions to assess the presence of genetically modified organisms in food," *Food Chemistry*, vol. 274, pp. 760–765, 2019.
- [9] N. Gryson, "Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review," *Analytical and Bioanalytical Chemistry*, vol. 396, no. 6, pp. 2003–2022, 2010.
- [10] C. Hupfer, H. Hotzel, K. Sachse, and K.-H. Engel, "Detection of the genetic modification in heat-treated products of Bt maize by polymerase chain reaction," *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*, vol. 206, no. 3, pp. 203–207, 1998.
- [11] T. Bauer, P. Weller, W. P. Hammes, and C. Hertel, "The effect of processing parameters on DNA degradation in food," *European Food Research and Technology*, vol. 217, no. 4, pp. 338–343, 2003.
- [12] E. Bergerová, Z. Hrnčířová, M. Stankovská, M. Lopařovská, and P. Siekel, "Effect of thermal treatment on the amplification and quantification of transgenic and non-transgenic soybean and maize DNA," *Food Analytical Methods*, vol. 3, no. 3, pp. 211–218, 2010.
- [13] E. Bergerová, Z. Godálová, and P. Siekel, "Combined effects of temperature, pressure and low pH on the amplification of DNA of plant derived foods," *Czech Journal of Food Sciences*, vol. 29, no. 4, pp. 337–345, 2011.
- [14] T. J. R. Fernandes, M. B. P. P. Oliveira, and I. Mafrá, "Tracing transgenic maize as affected by breadmaking process and raw material for the production of a traditional maize bread, broa," *Food Chemistry*, vol. 138, no. 1, pp. 687–692, 2013.
- [15] K. Bitskinashvili, I. Gabriadze, T. Kutateladze, B. Vishnepolsky, D. Mikeladze, and N. Datukishvili, "Effects of thermal-acid treatment on degradation and amplification of wheat and maize DNA," *Journal of Food and Nutrition Research*, vol. 57, no. 3, pp. 242–251, 2018.
- [16] F. Moreano, U. Busch, and K.-H. Engel, "Distortion of genetically modified organism quantification in processed foods: influence of particle size compositions and heat-induced DNA degradation," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 26, pp. 9971–9979, 2005.
- [17] K. R. Vijayakumar, A. Martin, L. R. Gowda, and V. Prakash, "Detection of genetically modified soya and maize: impact of heat processing," *Food Chemistry*, vol. 117, no. 3, pp. 514–521, 2009.
- [18] Z. Godálová, E. Bergerová, and P. Siekel, "Effect of high temperature and pressure on quantification of MON810 maize," *Czech Journal of Food Sciences*, vol. 31, no. 4, pp. 376–381, 2013.
- [19] R. V. Ballari and A. Martin, "Assessment of DNA degradation induced by thermal and UV radiation processing: implications for quantification of genetically modified organisms," *Food Chemistry*, vol. 141, no. 3, pp. 2130–2136, 2013.
- [20] Z. Hrnčířová, E. Bergerová, and P. Siekel, "Effects of technological treatment on DNA degradation in selected food matrices of plant origin," *Journal of Food & Nutrition Research*, vol. 47, no. 1, pp. 23–28, 2008.
- [21] C. A. M. Oliveira, C. M. Kommers, F. K. M. Lehmann, A. S. K. Fonseca, N. Ikuta, and V. R. Lunge, "Detection of genetically modified maize in processed products, dry grains, and corn ears intended for fresh consumption in South Brazil," *Genetics and Molecular Research*, vol. 15, no. 4, 2016.
- [22] N. Datukishvili, T. Kutateladze, I. Gabriadze, K. Bitskinashvili, and B. Vishnepolsky, "New multiplex PCR methods for rapid screening of genetically modified organisms in foods," *Frontiers in Microbiology*, vol. 6, p. 757, 2015.
- [23] T. Kutateladze, I. Gabriadze, B. Vishnepolsky, M. Karseladze, and N. Datukishvili, "Development of triplex PCR for simultaneous detection of maize, wheat and soybean," *Food Control*, vol. 34, no. 2, pp. 698–702, 2013.
- [24] B. Ehlers, E. Strauch, M. Goltz et al., "Nachweis gentechnischer Veränderungen in Mais mittels PCR," *Bundesgesundheitsblatt*, vol. 40, no. 4, pp. 118–121, 1997, in German.
- [25] I. Gabriadze, T. Kutateladze, B. Vishnepolsky, M. Karseladze, and N. Datukishvili, "Application of PCR-based methods for rapid detection of corn ingredients in processed foods," *International Journal of Nutrition and Food Science*, vol. 3, no. 3, pp. 199–202, 2014.
- [26] M. C. Samson, M. Gullí, and N. Marmiroli, "Multiplex real-time PCR assays for simultaneous detection of maize MON810 and GA21 in food samples," *Food Control*, vol. 30, no. 2, pp. 518–525, 2013.
- [27] F. Debode, E. Janssen, C. Bragard, and G. Berben, "Detection by real-time PCR and pyrosequencing of the *CryIAb* and *CryIAc* genes introduced in genetically modified (GM) constructs," *Food Additives & Contaminants: Part A*, vol. 34, no. 8, pp. 1398–1409, 2017.
- [28] A. Noguchi, H. Akiyama, K. Nakamura et al., "A novel trait-specific real-time PCR method enables quantification of genetically modified (GM) maize content in ground grain samples containing stacked GM maize," *European Food Research and Technology*, vol. 240, no. 2, pp. 413–422, 2015.
- [29] S. Wei, C. Wang, P. Zhu, G. Zhou, W. Fu, and X. Wu, "A high-throughput multiplex tandem PCR assay for the screening of genetically modified maize," *LWT*, vol. 87, pp. 169–176, 2018.

- [30] N. Datukishvili, I. Gabriadze, T. Kutateladze, M. Karseladze, and B. Vishnepolsky, "Comparative evaluation of DNA extraction methods for food crops," *International Journal of Food Science & Technology*, vol. 45, no. 6, pp. 1316–1320, 2010.
- [31] B. Vishnepolsky and M. Pirtskhalava, "ALIGN_MTX-An optimal pairwise textual sequence alignment program, adapted for using in sequence-structure alignment," *Computational Biology and Chemistry*, vol. 33, no. 3, pp. 235–238, 2009.
- [32] R. Kalendar, D. Lee, and A. H. Schulman, "Java web tools for PCR, in silico PCR, and oligonucleotide assembly and analysis," *Genomics*, vol. 98, no. 2, pp. 137–144, 2011.
- [33] J. F. Antiabong, M. G. Ngoepe, and A. S. Abechi, "Semi-quantitative digital analysis of polymerase chain reaction-electrophoresis gel: potential applications in low-income veterinary laboratories," *Veterinary World*, vol. 9, no. 9, pp. 935–939, 2016.
- [34] Y. Chen, Y. Ge, and Y. Wang, "Effect of critical processing procedures on transgenic components in quality and quantity level during soymilk processing of Roundup Ready soybean," *European Food Research and Technology*, vol. 225, no. 1, pp. 119–126, 2007.
- [35] F. Xing, W. Zhang, J. N. Selvaraj, and Y. Liu, "DNA degradation in genetically modified rice with *Cry1Ab* by food processing methods: implications for the quantification of genetically modified organisms," *Food Chemistry*, vol. 174, pp. 132–138, 2015.
- [36] Z. Nikolić, G. Petrović, D. Panković et al., "Threshold level and traceability of roundup ready® soybeans in tofu production," *Food Technology and Biotechnology*, vol. 55, no. 4, pp. 439–444, 2017.
- [37] B. M. Martin-Fernaandez, J. Costa, M. Beatriz, P. P. Oliveira, B. Lopez-Ruiz, and I. Mafra, "Combined effects of matrix and gene marker on the real-time PCR detection of wheat," *International Journal of Food Science & Technology*, vol. 51, no. 7, pp. 1680–1688, 2016.



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