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THE EFFECT OF HEAT PROCESSING ON PCR DETECTION OF GENETICALLY MODIFIED SOY IN BAKERY PRODUCTS

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Abstract:

Soy is commonly added to various foods because of its quality and health benefits. However, it is also the most commonly cultivated genetically modified (GM) crop. Hence, detection of GM soy in food preparations is an important goal of food science research. Although DNA is relatively stable during processing, and polymerase chain reaction (PCR) can be used to analyze processed food products, the processing factor-induced DNA degradation limits these methods. We evaluated the effect of different baking temperatures on the detection of GM soy in cookies by preparing cookies containing various amounts of GM soy and baking them at different temperatures and for different times. The effect of heat on the DNA quality was inspected by detecting the cauliflower mosaic virus 35S promoter and species-specific lectin sequences. As conclusion, the heating process affects the sensitivity of the PCR screening of GM organisms significantly, and the detection limit is elevated.

Keywords: GMO, PCR, GM soy, DNA degradation, Process factor

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Introduction

Since 1960s, soy and soy products have been used as ingredients in several food types. Addition of soy in food products not only improves the product quality (e.g., improved sensory characteristics and emulsification), but is also a valuable essential amino acid source. Several different forms of soy products can be used in the production process, such as soy flour/grits, soy protein concentrates, and soy protein isolates (Belloque et al., 2002). The product quality and health benefits of soy flour in bakery products have also been reported by many researchers (Alpaslan and Hayta, 2006; Singh et al., 2008). Singh et al. (2008) informed that addition of 2–5% defatted soy flour in hard cookies improves matching and produces a crispy texture. For this purpose, there has been a considerable interest in using soy derivatives in bakery products for many years.

However, despite all these advantages and large spectrum of use, soy is the first crop to be genetically modified (GM), and remains the main GM crop (James, 2011; Ujhelyi et al., 2008). Genetically modified organisms (GMOs) have come under harsh scrutiny since they were first commercialized. Several countries, including the Turkish Republic, set up official regulations enforcing the labeling of foods that contain GM materials above a threshold level (Miraglia, et al., 2004; Regulation (EC) No 1830/2003, 2003; Regulation (TR), 2010; Ujhelyi et al., 2008; Vijayakumar et al., 2009).

This enforcement prompted the food science area to develop reliable detection methods. The identification of novel DNA sequences or proteins is the main principle of such detection methods (Ahmed, 2002; Greiner and Konietzny, 2008; Lipp et al., 2000; Miraglia et al., 2004; Taski-Ajdukovic et al., 2009). However, because proteins are sensitive to most food processing factors, protein-based methods do not serve as sensitive and reliable tests for processed products in many cases (Ahmed, 2002, Bergerova et al., 2010). Because DNA is more resistant than proteins to such processes, DNA-based methods are more widely used for this purpose (Greiner and Konietzny, 2008; Taski-Ajdukovic et al., 2009). By using polymerase chain reaction (PCR), general GMO screenings and event-specific identifications or quantifications can be performed. Screening methods are based on the detection of common DNA elements, such as the cauliflower mosaic virus (CaMV) 35S promoter and/or the nopaline synthase (*nos*) terminator. In most cases, the detection strategy of GMOs in food starts with a general screening followed by event-specific identification and, if necessary, -quantification (Ahmed, 2002; Gryson et al, 2007; Miraglia et al., 2004). To achieve this strategy, there is the need for sensitive and reliable initial screening methods. There are several screening assays validated and introduced as standard methods (ISO 21569, 2005; Lipp et al. 2001). The major requirement for a successful screening with PCR is a sufficient quantity and amplifiable quality DNA (Bauer et al., 2003; Lipp et al., 2001; Peano et al., 2004; Tengel et al., 2001; Vijayakumar et al., 2009). However, most processing factors like low pH, heat processing, freezing, and drying affect the quality and quantity of the DNA and, thus, decrease the sensitivity of the test (Bauer et al. 2003; Gryson, 2010; Lipp et al., 2001; Murrayet al., 2009; Peanoet al., 2004; Tengel et al., 2001; Vijayakumar et al., 2009). Baking is known to affect negatively the results of PCR testing of GMOs in food products (Bauer et al., 2003; Gryson et al, 2007; Gryson, 2010; Straub, 1999). Therefore, the aim of our study was to evaluate the effect of baking at different temperature/time combinations on the PCR screening of novel (e.g., CaMV 35S) and species-specific (e.g., lectin) DNA sequences.

Materials and Methods

Model processed cookie production

Model cookies were produced from 225 g of wheat flour containing various amounts of GM soy, 64 g of margarine, 130 g of sugar, 33 g of dextrose solution (5.9%), 2.5 g of sodium bicarbonate, 2.1 g of salt, and 16 g of water, according to the approved method 10-50D (AACC International, 2000). The GM soy used for preparing the cookies was 1.25 and 2.5% Round Up Ready® (RUR) soy reference material (SDI diagnostics, USA). An appropriate amount of 2.5% RUR soy reference material (RM) was added to the wheat flour to give final concentrations of 0.1, 1.0, 3.0, and 5.0%. Model cookies containing 0.1 and 1% of 1.25% RUR soy were also prepared. The concentration of soy flour in the dough and the percentage of RUR soy in soy flour are detailed in Table 2.

The prepared dough samples were further dived into five subgroups. Each group was cut into 0.5 cm thick slices and cut into round cookie shapes. Four of these subgroups were baked at 170°C for

10 min, 170°C for 30 min, 200°C for 20 min, and 220°C for 15 min, respectively, while the fifth group was kept as raw dough (control group).

DNA extraction and purification

DNA was extracted in duplex from raw dough and model cookies using the Promega Wizard® Magnetic DNA Purification System for Food (Promega, Madison, USA) according to the manufacturer's instructions. Briefly, 1 g of sample material from a previously homogenized sample was mixed thoroughly with 2.5 mL of lysis buffer A® (Promega, Madison, USA) and 25 µL of RNAse A® (Promega, Madison, USA) and vortexed for 10 s. Then, 1.25 mL of lysis buffer B® (Promega, Madison, USA) was added and vortexed for another 10 s. Following incubation at 22–25°C for 10 min, 3.75 mL of precipitation solution® (Promega, Madison, USA) was added and centrifuged at 5000 × *g* for 10 min. The supernatant was transferred to a clean tube, mixed with 100 µL of Magnesil PMPs® (Promega, Madison, USA), and incubated at room temperature for 5 min with constant shaking. After addition of 0.8 volumes of isopropanol, the solid phase was captured in a magnetic separation stand and the liquid phase was discarded. The solid phase was washed once with 1.25 mL of lysis buffer B and three times with 5 mL of wash solution (70% ethanol) in the magnetic separation stand. After the solid phase was dried at 65°C for 10 min, 100 µL of nuclease-free water was added and incubated at 65°C for 5 min. The liquid phase (Genomic DNA) separated from this mixture in the magnetic separation stand, was collected in a clean tube and stored at −20°C until it was used.

The DNA concentration and purity of each extract were determined by UV-spectrophotometry at 260 and 280 nm using a T80 UV/VIS spectrometer (PG Ins. Ltd., UK). To evaluate the integrity of the DNA, 10 µL of the DNA extracts were subjected to electrophoresis in 1.5% agarose gel containing ethidium bromide.

PCR primers

The primers p35S-cf3 (5′-CCA CGT CTT CAA AGC AAG TGG-3′) and p35S-cr4 (5′-TTC TCT CCA AAT GAA ATG AAC TTC-C3′) that amplify a PCR fragment of 123 bp were used for screening PCR of the CaMV 35S sequence (ISO 21569, 2005). The primers Lectin 1 (5′-GAC GCT ATT GTG ACC TCC TC-3′) and Lectin 6 (5′- GAA AGT GTC AAG CTT AAC AGC GAC G-3′) were used for the amplification of soy-specific lectin sequence and yielded a longer PCR product (318 bp) (Tengel et al., 2001).

PCR conditions

All PCR reactions were performed with a CG Palm-Cycler (CG 1-96 Genetix Biotech, Australia and Asia). The amplification reactions contained 5 µL of genomic DNA (10 ng/µL) and 20 µL of the appropriate PCR reaction mixture. The PCR reaction mixture varied depending on the sequence: for CaMV 35S, it consisted of buffer (1× Fermentas), $MgCl_2$ (1.5mM; Fermentas), primers for CaMV 35S (0.6 µM), dNTPs (0.16 mM each; Fermentas), and MaximaTM Hot Start Taq polymerase (0.8 U; Fermentas); for soy-specific lectin, it consisted of buffer (1× Fermentas), $MgCl_2$ (2 mM; Fermentas), primers for lectin (0.5 µM), dNTPs (0.2 mM each; Fermentas), and MaximaTM Hot Start Taq polymerase (2 U; Fermentas) (ISO,21569, 2005; Tengel et al., 2001).

The amplification profiles used for these mixtures were as follows:

- For CaMV 35S: denaturation for 10 min at 95°C; amplification for 25 s at 95°C, 30 s at 62°C, and 45 s at 72°C; number of cycles 50; final extension for 7 min at 72°C.
- For lectin: denaturation for 3 min at 94°C; amplification for 45 s at 94°C, 45 s at 60°C, and 25 s at 72°C; number of cycles 50; final extension for 7 min at 72°C.

Agarose gel electrophoresis

The PCR products were electrophoresed through a 2% agarose gel containing ethidium bromide. A 50-bp DNA ladder (Sigma Chemical Co., St. Louis, MO) was used as size standard. The visualization of the gels was performed with a UV transilluminator, and the gels were captured with a Dolphin-DOC system and Dolphin 1D Gel analyzing software (Wealtec, Nevada, USA).

Results and Discussion

In this study, the effect of different levels and periods of baking on the PCR detection of soy-specific and GM DNA was evaluated. To achieve this aim, model cookies containing different levels (i.e., 0.1, 1, 3, and 5%) of 1.25% RUR or 2.5% RUR soy were prepared. These cookies were baked at 170°C for 10 min or 30 min, 200°C for 20 min, 220°C for 15 min, or left raw as a control.

For a successful PCR testing, the extraction of a sufficient quality and quantity of DNA is the first step (Ahmed, 2002; Gryson, 2010; Tengel et al., 2001). Several different extraction methods have been recommended for different food matrixes so far (Peano et al., 2004; Taski-Ajdukovic et al., 2009; Tengel et al., 2001). However, various factors such as the matrix type and processing conditions influence the performance of the extraction methods (Bergerova, et al., 2010; Gryson, 2010; Peanoet al., 2004). At the beginning of this study, we used the cetyltrimethylammonium bromide (CTAB) method, which had been modified to start with 1 g of sample (Ozgen Arun et al., 2013). However, because a quantifiable amount of DNA could not be extracted, we continued to use the Wizard® Magnetic DNA Purification System for Food. The manufacturer recommends two different protocols starting from 200 mg or 1000 mg of sample material. Since we could not obtain satisfactory amounts of DNA with this protocol when starting with 200 mg of the model cookie, we used the 1000 mg starting material protocol. Similarly, the results from other groups and our previous studies showed that increasing the sample weight allows for the extraction of a sufficient amount of DNA (Ozgen-Arun et al. 2013; Vijayakumar et al., 2009). Our study showed that a sufficient amount of DNA could be extracted by using this protocol. The mean DNA concentration that we obtained with the Wizard® Magnetic DNA Purification System for Food was 84.8-213 ng/µL (Table 1). These values were significantly higher from those reported by Bergerova et al. (2010), who reported that the DNA concentration they could obtain with the same method was 20-70 ng/µL. This difference may be attributable to the higher sample portion that we used.

To examine the effect of the baking time and temperature, agarose gel electrophoresis was performed on some of the DNA extracts (Figure 1). According to the results, raw dough showed a >3000 bp, clear, and distinct band (Figure 1, Lane 2). The model cookies baked at 170°C for 10 min showed a clear band with a lower integrity compared to the raw dough extracts and a smear at higher size compared to the DNA extracts of the samples baked at 170°C for 30 min and higher temperatures (Figure 1, Lanes 3 and 4). Following electrophoresis of DNA extracts of the cookies baked at 200 and 220°C, the large and clear bands were replaced by a strong smear, thus indicating deterioration (Figure 1, Lanes 5 and 6).

To evaluate the effect of baking on the quantity of DNA, the DNA concentration in extracts was calculated by using the 260 nm absorbance values. The concentrations of the raw dough and cookie samples baked at different times and temperatures are summarized in Table 1. Surprisingly, the DNA concentration was the highest in the extracts of the cookies baked at 170°C for 30 min. The DNA levels of the extracts constantly decreased with the increase of the baking temperature. Previously published results support these findings. According to Pauli et al. (2000), the DNA concentration in highly processed soy samples was higher. Bergerova et al. (2010) also determined that the DNA concentration of soy flour boiled for 30 min was higher than that of samples boiled for 7 and 15 min. Their results also showed that the DNA concentration decreased in parallel with the boiling time. The lower DNA concentration of raw dough is most probably related to the physical structure of the sample matrix. In fact, it is not possible to obtain the same type of fine powder for extraction from dough-like cookies. Indeed, other studies reported that the particle size of the sample strongly affects the DNA extractability (Begerova et al., 2010; Moreano et al., 2005).

Table 1. The absorbance readings, concentration and purity of the DNA extracts

Temperature/Time	260 nm*	280 nm*	Purity*	Conc.* (ng/µl)
Raw	0.12	0.09	1.34	144.5
170ºC/10 min	0.07	0.05	1.26	84.8
170ºC/30 min	0.17	0.14	1.26	213
200ºC/20 min	0.14	0.13	1.06	178
220ºC/15 min	0.09	0.08	1.10	104

* The values are mean of all soy concentrations

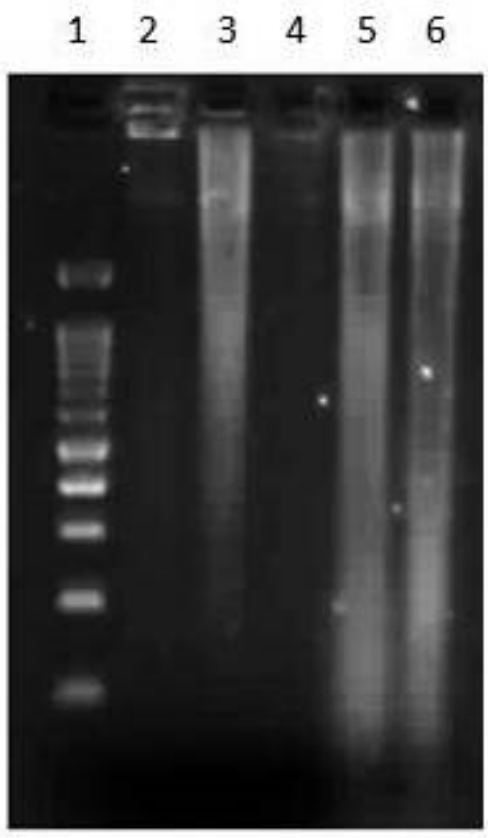

Figure 1. Agarose gel electrophoresis of the DNA extracts; Lane 1: DNA ladder (50-3000bp), Lane 2: Dough, Lane 3: 170°C 10 min, Lane 4: 170°C 30 min, Lane 5: 200°C 20 min, Lane 6: 220°C 15 min

The purity of the extracted DNA was assessed by measuring the A_{260}/A_{280} UV absorbance ratios (Gryson, 2010). Although the purity values of the extracts that we obtained were between 1.06 and 1.34, the detection of the lectin sequence in all the extracts proved that they contain amplifiable quality DNA. Similar to our results, the purity values of the DNA extracts that Bergerova et al. (2010) obtained from baked soy flour samples were between 1.10 and 1.38.

In most cases, routine GMO detection strategy starts with the general screening of GMOs in the product. For this purpose, sensitive and accurate screening is an important necessity for obtaining reliable results. Therefore, we evaluated the applicability of a CaMV 35S screening assay on baked food products. Because most routine laboratories implementing the ISO 17025 accreditation requirements prefer interlaboratory validated standard methods and need to verify them further for different food matrixes, we preferred to use the primers suggested in the standard methods (ISO 17025, 2005; ISO 21569, 2005).

We performed verification and quality control tests on the primers that we used in our study. To verify the lectin primers, we performed a PCR with RUR soy-containing model cookies, dough without soy flour, and RUR soy certified reference materials (CRMs), and confirmed that the primers were specific to soy DNA (Figure 2). The sensitivity of the CaMV 35S assay used in our study was determined by testing 0.1, 0.5, and 1% RUR soy CRMs. Positive detection of 0.1% RUR soy CRM proved that the detection limit of the method was not above 0.1% (Figure 3). Appropriate controls were used during all the PCR tests performed in this study; a PCR setup without template DNA (sterile Milli Q water) in every PCR test was used as the negative control to eliminate false positive results related to contamination. Additionally, 0% RUR soy CRM was used as the negative control in all CaMV 35S PCR set-ups. In every PCR set-up, 0.1, 0.5, and 1% RUR soy CRM was used as the positive control.

During our study, PCR tests were repeated to obtain four amplification results from each sample, which were extracted in duplicate, for both lectin and CaMV 35S sequences. The results are summarized in Table 2. According to these results, 5% RUR soy could only be detected when the cookies were baked at 170°C for 30 min and 200°C for 20 min, in two out of four repeats. However, the results of baking at 220°C for 15 min showed that RUR soy could be detected in all the repeats of 5% and two out of four repeats of 3%. This suggested that the exposure time is also an important variable affecting the detectability and a lower detection limit can be obtained even at higher temperatures. The results of 10 min heating at 170°C supported these findings. Although the detection could be possible in only two out of four repeats after 30 min baking at 170°C in 5% soy cookies, 100% detection could be obtained from cookies containing 1% soy or above concentrations and 50% detection from cookies containing 0.1% soy after 10 min baking at 170°C. This finding complies with the results of Bergerova et al. (2010), who found that the integrity of DNA in soybean samples baked at 220°C significantly decreased with time. Similarly, some other studies also indicated the importance of the treatment time on GMO detection (Grayson, 2010; Vijayakumar et al., 2009).

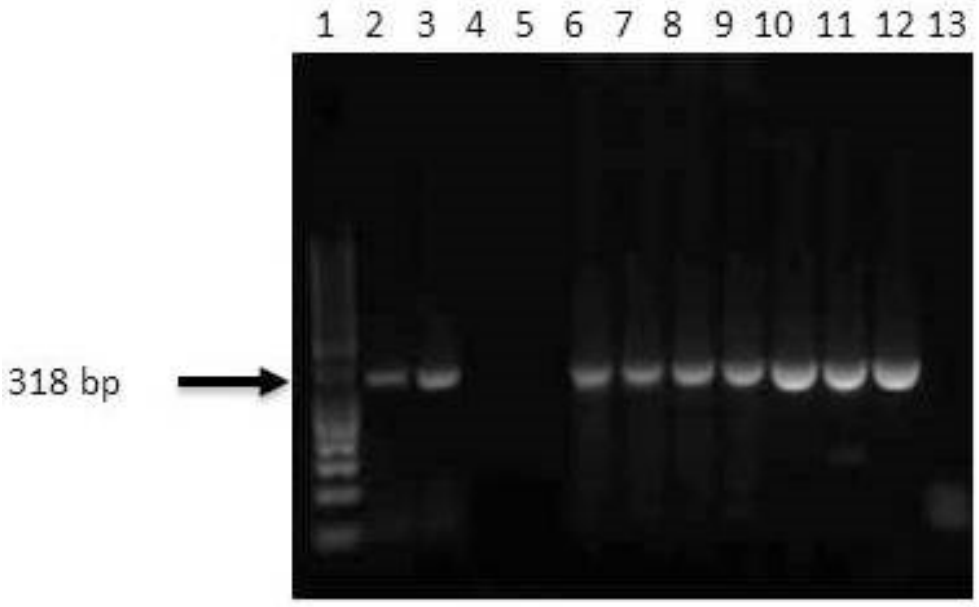

Figure 2. Agarose Jel Electrophoresis of Lectin PCR Lane 1: 50 bp DNA ladder, Lane 2-3: Cookie (5% soy), Lane 4-5: Cookie (without soy), Lane 6-7: Cookie (1% soy), Lane 8-9: Cookie (3% soy) Lane10-12: RUR soy CRMs, Lane 13: PCR negative (Sterile milli Q water)

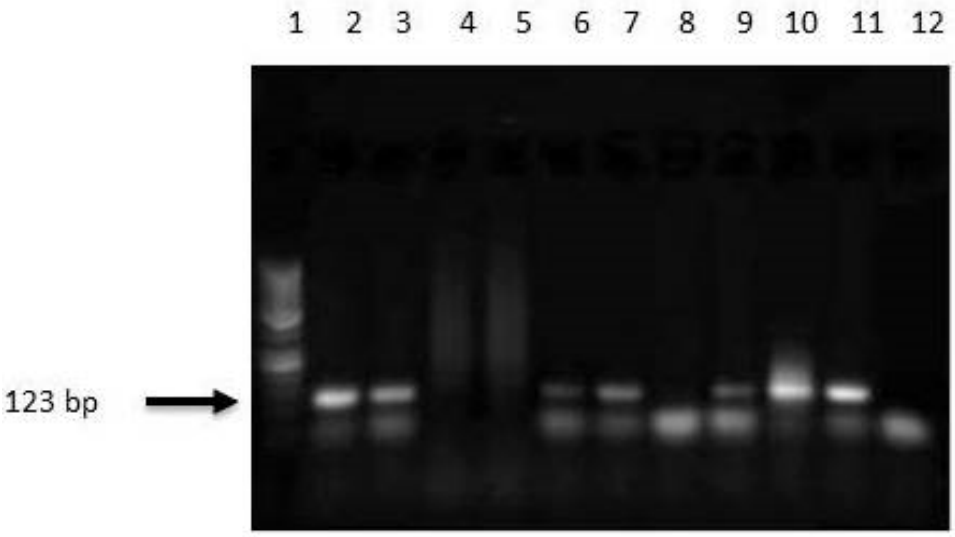

Figure 3. Agarose Jel Electrophoresis of CaMV 35S PCR Lane 1: 50 bp DNA ladder, Lane 2-3: Dough (5% soy containing), Lane 4-5: Cookie (5% soy 200ºC baked), Lane 6-7: Cookie (5% soy 220ºC baked), Lane 8-11: RUR soy CRMs (0, 0.1, 0.5 ve 1%) Lane 12: PCR negative (Sterile milli Q water)

Table 2. PCR screening results of model samples determined with primer pairs for CaMV 35S and lectin sequences

Total Soy/Dough	RUR Soy / Total Soy	Temperature/Time	Results*	
			35 S	Lectin
0.1%	1.25%	Raw	2	4
		170ºC/10 min	2	4
		170ºC/30 min	0	4
		200ºC/20 min	0	4
		220ºC/15 min	0	4
	2.50%	Raw	2	4
		170ºC/10 min	2	4
		170ºC/30 min	0	4
		200ºC/20 min	0	4
		220ºC/15 min	0	4
1%	1.25%	Raw	4	4
		170ºC/10 min	4	4
		170ºC/30 min	0	4
		200ºC/20 min	0	4
		220ºC/15 min	0	4
	2.50%	Raw	4	4
		170ºC/10 min	4	4
		170ºC/30 min	0	4
		200ºC/20 min	0	4
		220ºC/15 min	0	4
3%	2.50%	Raw	4	4
		170ºC/10 min	4	4
		170ºC/30 min	0	4
		200ºC/20 min	0	4
		220ºC/15 min	2	4
5%	2.50%	Raw	4	4
		170ºC/10 min	4	4
		170ºC/30 min	2	4
		200ºC/20 min	2	4
		220ºC/15 min	4	4

* The number of positive results in 4 repated PCR

In contrast to the results obtained for the baked samples, 100% amplification of the CaMV 35S sequence could be obtained from 1, 3, and 5% dough samples. When the soy flour ratio in the dough was lowered to 0.1%, only 50% positive amplification reactions could be obtained.

The results of the lectin PCR proved that all the model cookies had sufficient amounts of amplifiable soy DNA and the negative results are true negative. Accordingly, 100% amplification could be performed from all the samples, irrespective of the soy flour ratio and processing conditions, even though the target fragment length necessary for the lectin assay (318 bp) is significantly longer than that for the CaMV 35S assay (123 bp). Similarly, other researchers also reported that the processing conditions have different effects on the endogenous and exogenous genes of Roundup Ready soy (Bergerovaet al., 2010; Chen et al., 2005). The other possibility would be the relatively lower ratio of GM soy to total soy. Although Vijakumar et al. (2009) reported that the detection limit increased with the increase of the ratio of RUR soy in total soy flour, the results for the 1.25% and 2.5% RUR soy samples were not different in our study. However, the ratios used in our study are very close and do not totally eliminate the aforementioned possibility. Thus, considering the high importance of GMO quantification, further studies should be conducted. The regulations on GMOs require the labeling of foods containing GM material above 0.9% (Regulation (TR), 2010; Regulation (EC) 1830/2003, 2003).

The thresholds given here are for the portion of the GM-specific gene sequence in respect to the reference gene (Gryson, 2010). Therefore, the detection limit of the method should be low enough to detect low levels of GM material even when the GM-specific gene ratio is as low as 1%.

Our results showed that there is no correlation between the effect of processing on the DNA concentration in the extract and the detectability of GMOs by PCR. Although the DNA concentration was the lowest in the extracts of cookies baked at 170°C for 10 min, the highest detection limit (100% detection in 1% RUR soy and 50% in 0.1% RUR soy cookie samples) was obtained from these extracts.

Conclusion

In conclusion, processing techniques strongly affect the results of PCR testing. The results of our study indicated that, although amplifiable quality and quantity of DNA could still be obtained from a processed and complex food matrix such as that in a cookie, baking has an important effect on the detectability of GM soy in food samples by PCR and the detection limit of the method was significantly elevated. Besides, our results also proved that not only the temperature itself, but also the exposure time to heat is an important factor. Nevertheless, it was also determined that the effect of baking on endogenous and exogenous genes might be different and that endogenous genes could be more stable under the processing conditions. This finding has to be further studied in detail because of its ability to affect the accuracy of quantitative methods.

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