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RESEARCH ARTICLE

The Effect of Heat Processing and pH on PCR Detection of Genetically Modified (GM) Soy in Meat Products [1]

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Summary

Soybean is the most cultivated GM crop worldwide, being planted on 47% of the global GM crop area. However, GM crops have not been widely publicly accepted. Thus, countries have established regulations for the labeling of GM foods to inform consumer decision making. In 2010, regulations on GM foods began to be enforced in Turkey. To meet these legislation requirements, the development of reliable detection methods is an important priority in this research area. PCR-based methods are most commonly used for this purpose. However, processing factors (low pH, heat etc.) affect DNA quality and thus the sensitivity of PCR. The aim of this study was to evaluate the combined effects of heat and pH on the detection of GM soy in meat products. We found that the combined effects of heat and low pH affect the detection limit, but low levels of GM soy can still be detected after processing.

Keywords: *GMO, PCR, Meat products, GM soy, DNA degradation, Process factor*

Et Ürünlerinde Isıl İşlem Uygulamaları ve pH'nın Genetiği Değiştirilmiş (GD) Soyanın PCR ile Tespiti Üzerine Etkisi

Özet

Soya fasulyesi dünya genelinde en yaygın ekilen GD tahıl ürünü olup toplam GD tarım üretim alanının %47'sini teşkil etmektedir. Buna karşın, GD tarım ürünleri tüketiciler tarafından yaygın olarak kabul görmemektedir. Bu yüzden, çeşitli ülkeler tüketicilere karar verme olanağı tanımak üzere GD gıdaların etiketlemesine yönelik yönetmelikler hazırlamıştır. Nihayetinde, 2010 yılında ülkemizde de GD gıdalarla ilgili mevzuat yürürlüğe girmiştir. Yönetmelik şartlarını karşılamak için, güvenilir tespit metotlarının geliştirilmesi bu bilim alanının önceliklerinden biri haline gelmiştir. Bu amaçla PCR-temelli metotlar en yaygın kullanılan teşhis yöntemlerindendir. Ancak, çeşitli gıda üretim yöntemleri (düşük pH, sıcaklık v.b.) DNA kalitesini ve böylece PCR hassasiyetini etkilemektedir. Çalışmamızın amacı, ısı ve pH'ın et ürünlerinde GD soyanın PCR tespitine olan kombine etkisini değerlendirmektir. Sonuçlarımıza göre, düşük pH ve ısının kombine etkisi tespit limitini etkilemekle beraber, proses sonrası dahi oldukça düşük seviyelerde GD soyanın tespiti mümkün olabilmektedir.

Anahtar sözcükler: *GMO, PCR, Et ürünleri, GD soya, DNA yıkımlanması, Proses etkisi*

INTRODUCTION

Soy is an important crop for the food industry because it is widely used in many foods [1-3]. In the case of meat products, soy protein is a common ingredient because of its unique functional properties, such as water and fat binding capabilities and the ability to improve organoleptic features. Furthermore, soy protein is also an economic protein replacement that can reduce production costs [2-5]. However, soy is also important for being the first commercial GM crop (Roundup Ready (RR) soy). It was developed by the Monsanto Company and is still the most cultivated GM crop; presently, it accounts for 47% of the global GM crop area [6,7].

Similar to several other countries, Turkish food regulation also enforces the labeling of foods that contain approved GM material above a threshold level [7-14]. To meet these regulation requirements, various studies have been performed to develop reliable and sensitive detection methods [1,15,16]. PCR is the most common method used for this aim [3,17,18]. By using PCR, general GMO screening

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and event-specific identification can be performed. Screening methods that 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, are generally the initial step in GMO detection (before event identification or GMO quantification); therefore, their reliability is important for most users [1,11,19-21]. Thus, screening assays based on the detection of CaMV 35S and *nos* sequences have been interlaboratory validated, and the method has been accepted as an official standard method [22-25].

Although DNA is more stable than protein in processed foods, it can still be degraded under processing conditions [14,25-29]. Because PCR-based detection of GMOs depends on the quality, purity and quantity of DNA, degradation reduces the sensitivity of analysis and has a negative impact on the detection limit of the method [30,31]. Temperature and pH are known to be the most contributory factors to DNA fragmentation [26,30]. In several studies, the degradation effect of various heating processes (drying, cooking, baking, autoclaving and spray-drying) commonly used for food production were evaluated. The results of all these studies proved that heat processing of foods caused mild to strong fragmentation of DNA and thus limited the ability to perform PCR screening [16,20,31-34]. Additionally, Bauer *et al.*[26], found that the highest DNA degradation occurred due to a combined exposure to acidic conditions and heat. In that study, researchers lowered the pH of soy flour with acetic acid and extracted DNA from these samples. The researchers also informed that the stability of DNA in different food matrices would also be different because each food processing technique and matrix would led to a unique environment. Gryson [30], also explained the importance of the effects of the type of food matrix on the performance of DNA extraction and PCR testing.

Therefore, in this study, we aimed to evaluate the combined effects of pH and heat, which are the most common processing factors used in meat production, on the PCR screening of GMOs.

MATERIAL and METHODS

Certified Reference Materials and Food Samples

Certified reference materials (CRMs) consisting of soybean powder (0, 0.1, 0.5 and 1% of RR soybean powder) produced by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) and commercial soy containing meat sausage, soy flour and soy-free bread samples were used for quality control and verification purposes in the study.

Model Processed Sausage Production

Model processed sausages were produced from a formula of a commercial meat product producer in Turkey. The sausage mixtures were prepared from 390 g of beef, 160 g of fat emulsion, 340 g of ice, 40 g of oil, 0.05 g of paprika, 0.15 g of nitrate, 15 g of salt, 15 g of mixed spices (ginger, white pepper), 40 g of potato starch, and 0.20 g of carmine. Then, the appropriate amount of 1.25% RR soy (SDI diagnostics, USA) was added to the sausage mixtures to give final concentrations of 0.1, 0.5 and 1%. Each of the model sausage mixtures were divided into two groups, and the pH of these groups were adjusted to either 5.2 or 6.2. Following pH adjustment, each group was further divided into three subgroups, two of which were heated for 15 min at either 65°C or 85°C, while the third subgroup was left untreated (control group) *(Fig. 1)*.

DNA Extraction and Purification

DNA was extracted and purified in duplex from raw and heated model sausages, CRMs and food samples, using a Promega Wizard™ DNA isolation kit (Promega, Madison,

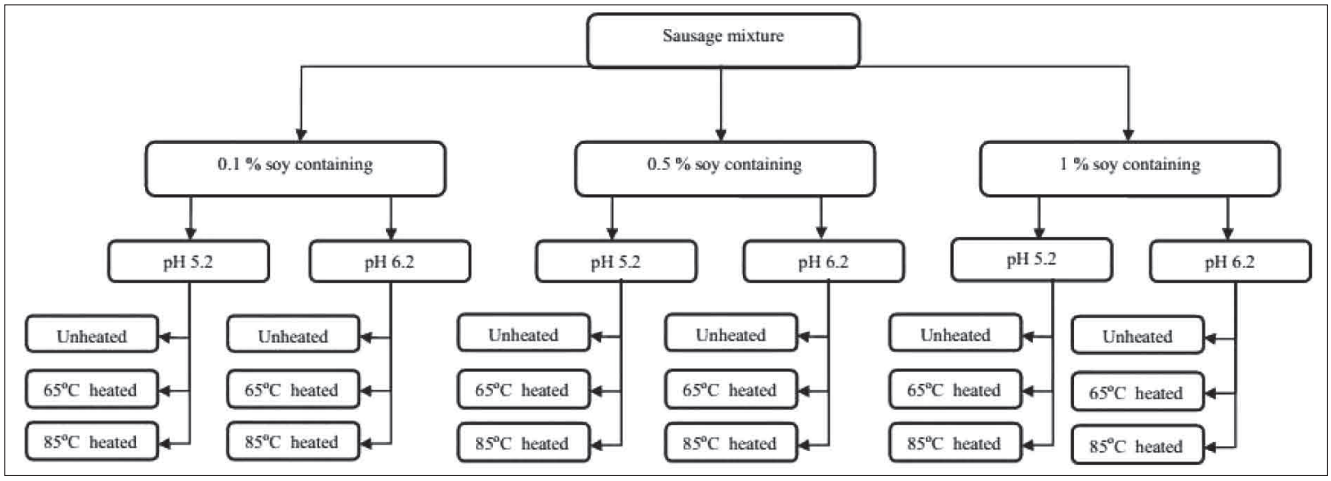

Fig 1. Model processed sausage production
Şekil 1. Model sosislerin üretimi

USA) according to the manufacturer's instructions and as described in other studies [17,23]. Briefly, between two hundred to three hundred milligrams of food material from a previously homogenized sample was mixed with 860 µl of extraction buffer (10 mM Tris-OH, 150 mM NaCl, 2 mM EDTA and 1% w/v sodium dodecyl sulfate), 100 µl of guanidine hydrochloride (5 M) and 40 µl of proteinase K (20 mg/ml) and then incubated at 65°C overnight. The samples were then centrifuged at 13.500 g for 10 min. After centrifugation, 500 µl of supernatant was mixed with 1 ml of Wizard™ resin (Promega, Madison, USA) and pushed through a Wizard™ minicolumn (Promega, Madison, USA). The column was further washed with 2 ml of isopropanol. Following centrifugation of the column at 12.000 g for 5 min, DNA was eluted with 50 µl of pre-warmed (65°C) elution buffer (10 mM Tris-OH). The columns were incubated at room temperature for 1 min and centrifuged at 10.000 g for 2 min. The collected DNA was stored at -20°C until it was used.

The quantity and purity of the DNA were monitored by measuring the UV absorption at 260 nm and 280 nm using a T80 UV/VIS spectrometer (PG Ins. Ltd., UK). The integrity of the DNA was verified by loading the DNA onto a 2% 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 [22]. 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 amplification of soy specific lectin sequence and yielded a longer PCR product of 318 bp [28].

PCR Conditions

All PCR reactions were performed with a CG Palm-Cycler (CG 1-96 Genetix Biotech, Australia & 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 was varied: for the CaMV 35S amplifications, it consisted of 1X buffer (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 0.6 µM primers for 35S, 0.16 mM aliquots of each dNTP (Fermentas) and 0.8 U of Maxima™ Hot Start *Taq* polymerase (Fermentas); for soy-specific lectin amplifications, it consisted of 1X buffer (Fermentas), 2 mM $MgCl_2$ (Fermentas), 0.5 µM primers for lectin, 0.2 mM aliquots of each dNTP (Fermentas) and 2 U of Maxima™ Hot Start *Taq* polymerase (Fermentas) [22,28].

The amplification profiles used for these mixtures were as follows:

- For CaMV 35 S: denaturation for 10 min at 95°C; amplification for 25 s at 95°C, for 30 s at 62°C and for 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, for 45 s at 60°C and for 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. As a size reference, a 50 bp DNA ladder (Fermentas) was used. Visualization of the gels was performed with a UV trans-illuminator, and the gels were captured with a Dolphin-DOC system and Dolphin 1D Gel analyzing software (Wealtec, Nevada, USA).

RESULTS

Verification and Quality Control Testing

The PCR conditions and primers were verified in the beginning of the study. For this, a lectin PCR with soy containing sausage samples, soy-free bread sample, soy flour and RR soy CRMs was performed. The results are given in *Fig. 2*. According to these results the primers used in the assay generated PCR product only with the food samples containing soy and did not generate any amplification with non-soy containing bread sample.

The results of verification of CaMV 35S assay with CRMs are given in *Fig. 3*. These results also showed that the primers generate amplification with RR soy CRMs even when the GM soy content is as low as 0.1% and did not generate any PCR products with non GM soy CRM (0% RR soy CRM).

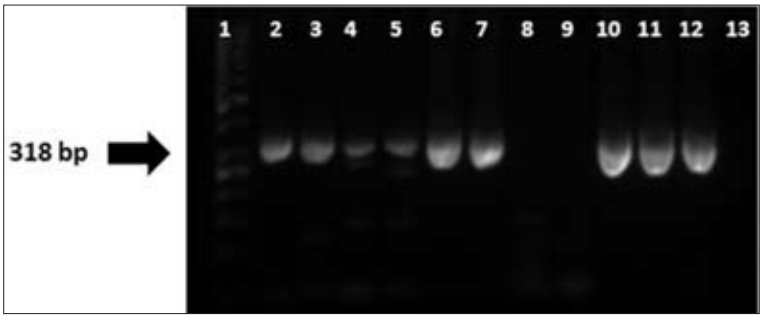

Fig 2. Lectin PCR: Lane-1:50 bp DNA ladder, Lane 2-5: Soy containing sausages, Lane 6-7: Soy flour, Lane 8-9: Soy free bread sample, Lane 10-12: RR soy CRMs Lane-13: PCR milli q water

Şekil 2. Lektin PCR: 1. Sıra: 50 bp DNA marker, 2-5. Sıra: Soya içeren sosis örnekleri, 6-7. Sıra: Soya unu, 8-9. Sıra: Soya unu içermeyen ekmek örneği, 10-12. Sıra: RR soya SRM'leri, 13. Sıra: PCR milli q su

Quality and Quantity of Extracted DNA

The results of agarose gel electrophoresis performed with DNA extracts of the model samples are given in *Fig. 4*. Although both groups showed a band above 1.000 bp (The highest band of the DNA ladder is 1.000 bp), the band intensities of DNA extracted from pH 5.2 sausages were significantly lower compared to the intensity of the DNA bands from the pH 6.2 samples. The DNA concentration and purity ratios which were calculated from the 260 nm absorbance readings and 260/280 absorbance reading ratios of the extracts respectively are detailed in *Table 1*. According to the results of our study, the overall average DNA concentrations were 116 ng/µl and 119 ng/µl for pH 5.2 and pH 6.2 sausages, respectively. The overall mean purity ratio of DNA extracts of pH 5.2 sausages was 1.51, while it was 1.39 for pH 6.2 sausages *(Table 1)*.

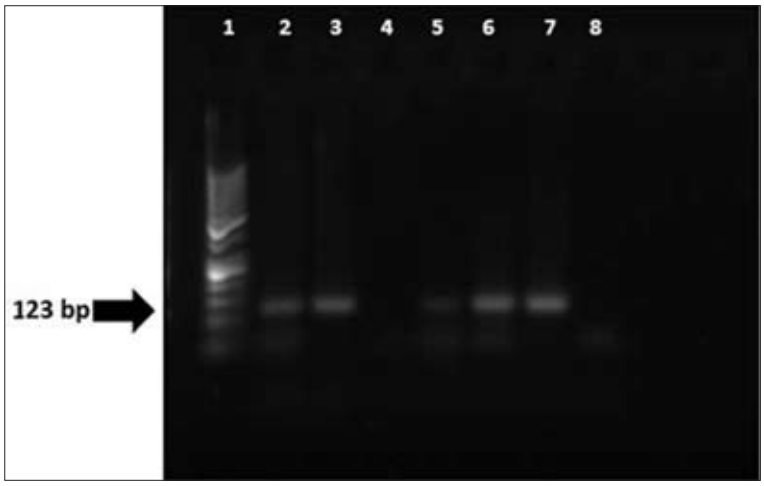

Fig 3. CaMV 35S PCR: Lane-1:50 bp DNA ladder, Lane 2-3: Heated model sausage (0.5%, pH 6.2, 85ºC), Lane 4-7: RR soy CRMs 0%, 0.1%, 0.5% and 1%, Lane-8: PCR milli q water

Şekil 3. CaMV 35S PCR: 1. Sıra: 50 bp DNA marker, 2-3. Sıra: Isıl işlem görmüş model sosis örnekleri (%0.5, pH 6.2, 85ºC), 4-7. Sıra: RR soya SRM'leri %0, %0.1, %0.5 ve %1, 8. Sıra: PCR milli q su

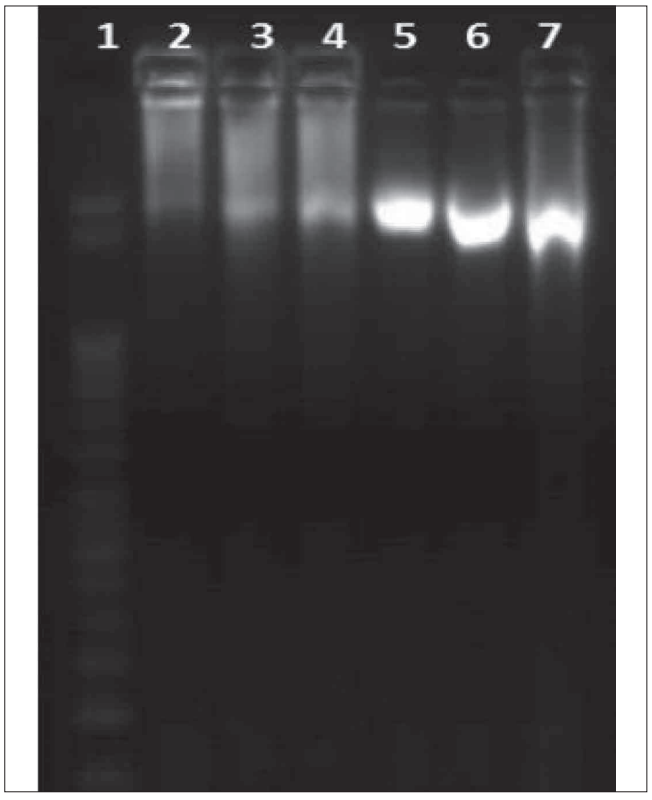

Fig 4. Agarose gel electrophoresis of the DNA extracts of model sausages; Lane-1:50 bp DNA ladder, Lane 2: pH 5.2 raw, Lane 3: pH 5.2 65ºC, Lane 4: pH 5.2 85ºC, Lane 5: pH 6.2 raw, Lane 6: pH 6.2 65ºC, Lane 7: pH 6.2 85ºC

Şekil 4. Model sosis örneklerine ait DNA ekstarktlarının Agar Jel Elektroforezi; 1. Sıra: 50 bp DNA marker, 2. Sıra: pH 5.2 çiğ, 3. Sıra: pH 5.2 65ºC, 4. Sıra: pH 5.2 85ºC, 5. Sıra: pH 6.2 çiğ, 6. Sıra: pH 6.2 65ºC, 7. Sıra: pH 6.2 85ºC

PCR Testing

Duplex extractions were made from the model sausage samples, and PCR tests were then repeated to obtain four amplification results for each sample for both the lectin and the CaMV 35S sequences. The results of the replicated amplifications are summarized in *Table 2*. The results of CaMV 35S PCR detection showed that when the ratio of 1.25% RR soy in the sausage sample was 0.5% or higher, 100% (4/4) positive amplification of GM-specific sequences was observed in raw, 65°C- and 85°C-heated samples from both pH 5.2 and 6.2 sausages. However, when the content of 1.25% RR soy was lowered to 0.1%, positive amplification could only be obtained from 50% (2/4) of the pH 6.2 samples. In the pH 5.2 sausages, detection was not possible when the sausage was heated at 85°C, while 50% detection was achieved for samples heated at 65°C.

Parallel to the PCR screening of RR soy, PCR testing for amplification of the lectin sequence was also performed *(Table 2)*.

According to these results, 100% amplification could be performed from all the samples, irrespective of the soy flour ratio and processing conditions.

DISCUSSION

The effects of processing factors on the quality and quantity of extracted DNA have also been studied by

Table 1. *The concentration and purity of the DNA extracts*
Tablo 1. *DNA ekstraktlarının konsantrasyon ve saflığı*

Sample Type		DNA Concentration (ng/µl)	Mean DNA Concentration	Purity	Mean Purity
5.2	Unheated*	108	116	1.69	1.51
	65°C*	93		1.38	
	85°C*	146		1.47	
6.2	Unheated*	112	119	1.43	1.39
	65°C*	137		1.44	
	85°C*	108		1.30	

** The concentration and purity are mean of 0.1, 0.5 and 1% samples*

Table 2. *PCR screening results of model samples determined with primer pairs for CaMV 35S and lectin sequences*
Tablo 2. *Model sosis örneklerinin CaMV 35S ve lektin dizilimleri için primer çiftleri ile gerçekleştirilen PCR tarama test sonuçları*

1.25% RR Soy/mixture	pH	Heat	CaMV 35S*	Lectin*
0.1%	5.2	Unheated	2/4	4/4
		65°C	2/4	4/4
		85°C	0/4	4/4
	6.2	Unheated	2/4	4/4
		65°C	2/4	4/4
		85°C	2/4	4/4
0.5%	5.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4
	6.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4
1%	5.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4
	6.2	Unheated	4/4	4/4
		65°C	4/4	4/4
		85°C	4/4	4/4

** The number of positive results in 4 repeated PCR*

other researchers; in these studies, heating was evaluated for certain food types [20,32-34]. However, because the food matrix would also affect the extractability and amplifiability of the DNA, studies should also be performed on various food types. In this study, the possibility of detecting low levels of GM soy in processed meat products by PCR is evaluated. For this purpose, model processed sausages containing various levels (0.1, 0.5 and 1%) of 1.25% RR soy were prepared. The pH of the products was adjusted to 6.2 or 5.2, and both groups were further divided into subgroups that were heated at 65°C or 85°C for 15 min or left unheated as a control.

The results of the verification PCRs proved that primer pairs used in the study are specific to target DNA and do not generate any amplification with non-target DNA (*Fig. 2* and *3*). The sensitivity of the CaMV 35S screening assay was determined by testing 0.1, 0.5 and 1% RR soy CRMs in parallel with the samples in each PCR. Positive detection of 0.1% RR soy CRM proved that the detection limit of the CaMV 35S assay is below 0.1% *(Fig. 3)*. For elimination of any false positive results, a no template control (sterile MILLI Q water) was run in each lectin and CaMV 35S specific PCR and 0% RR soy CRM was run in each CaMV 35S PCR [35].

Many other researchers have shown that a sufficient quality and quantity of DNA is necessary for successful PCR testing [19,28,30]. Additionally, processing technologies and extraction methods are crucial for maintaining the integrity of extracted DNA [16,20,26,30]. For this purpose, the effects of temperature and pH on the integrity of DNA extracted from raw and processed model sausages were examined by loading some of the DNA extracts onto a 2% agarose gel *(Fig. 4)*. These results proved that, although

DNA of low pH samples had a lower intensity, DNA fragments of sufficient length are still present, indicating that PCR should be possible. The DNA concentration in the extracted DNA which were determined by measuring the absorbance at 260 nm showed that the DNA concentrations did not indicate a significant difference between low and high pH sausages and different heating temperatures *(Table 1)*. The purity of the DNA extract is reported as another important variable that has an effect on PCR detection. The purity of the extracted DNA can be determined by measurement of A260/280 absorbance ratios with a spectrophotometer. DNA extracts are considered "suitable for PCR" when the ratio is between 1.5 and 2.0 [30]. Although the mean ratio of the DNA extracts from some of the model sausages were out of the purity ratio recommended for PCR in our study, lectin and CaMV 35S sequences could still be amplified from these extracts. Similarly, the results of Kakihara *et al.*[16], showed that although the 260/280 nm absorbance ratios of DNA solutions extracted using an alkaline lysis method were as low as 1.2, they could detect PCR products of 100 to 150 bp fragments from these solutions.

Screening PCR is reported to be the most sensitive PCR in GMO analysis, and it is generally used as the first step in GMO detection before both event identification and GMO quantification in routine monitoring [20,23]. Therefore, we used the CaMV 35S screening method to determine the effects of temperature and pH on the ability of GM DNA to serve as a template for amplification. Because most testing laboratories prefer internationally validated standard methods for routine testing, as recommended in the ISO 17025 laboratory accreditation standard, the primers used in the standard method for amplifying the CaMV 35S promoter were also used in this study [22,36].

According to the results of CaMV 35S PCR, detection was possible, when the ratio of 1.25% RR soy in the sausage sample was 0.5% or higher, after all types of processing conditions. The decrease in the ratio of the (2/4) positive results obtained from pH 6.2, 0.1% RR soy containing sausage samples was most likely related to the low RR soy content. The lack of amplification in 85°C heated pH 5.2 samples while it was present in 85°C heated pH 6.2 samples proved that heat processing more strongly effects the detection in lower pH matrixes. The amplification of the lectin sequence in the same extracts proved that negative results were not related to the absence of amplifiable quality soy DNA but, rather, to the practical detection limit of the method. Similarly, the results of Gryson *et al.*[20], showed that the practical detection limit of GM soy screening in cooked model cookies was higher compared to raw dough. The results obtained for sausages at pH 5.2 and heated to 85°C agreed with the results of Bauer *et al.*[26], who also showed that the combined effects of pH and heating were stronger than either effect alone.

Because lectin is present in both GM and non GM soy, PCR testing for amplification of the lectin sequence was also performed for evaluating homogeneity of the sample and amplifiability of the DNA extract. Additionally, information about the effects of processing on endogenous and exogenous DNA is also valuable for quantitative PCR testing. Even though the target fragment length necessary for the lectin assay is significantly longer than the CaMV 35S assay used in this study, detection could be possible from all samples. Similarly, other results showed that processing conditions have different effects on endogenous and exogenous genes of Roundup Ready soy [33]. However, the relatively lower ratio of GM soy (1.25% RR soy) in the final product might also be a reason.

In conclusion, the efficiency of a PCR detection method strongly depends on the quality, quantity and amplifiability of the DNA extract, which is affected by processing techniques. Our results proved that the effect of low and high temperature pasteurization processing used for meat products does not have a very strong effect on GMO screening and can be used to a certain extent. However, it was also determined that the combined effects of pH and heating are stronger than either individually, and the method should be further evaluated for low pH meat products heated at higher temperatures. The effect of processing endogenous and exogenous genes seemed to be different, and further studies into this concept should be performed because of its important effect on the accuracy of quantitative methods.

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