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RESEARCH ARTICLE

A comparison of DNA extraction methods and PCR-based detection of GMO in textured soy protein

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Abstract

Monitoring the presence of genetically modified organisms (GMOs) in a variety of food is important to many countries, as the law requires that the approved GMOs should be labeled as such. In addition, before genetically modified crops are used to obtain feed for the livestock, tests must be carried out to screen unapproved genetically modified varieties. Therefore, it is necessary to be able to detect and accurately quantify the amount of transgenic material present in food and feed. The analysis of processed soybean used in food and feed involves a number of complications, which negatively affect the DNA extraction. Therefore, the successful selection of DNA extraction methods is important for the detection of specific DNA targets in textured soy protein (TSP). The aim of this study was to compare three methods of DNA extraction from TSP, namely CTAB, modified CTAB and phenol/Chloroform methods. To this end, polymerase chain reaction (PCR) method was used to monitor products derived from GMOs, which specifically amplify the *35S* promoter, *NOS* terminator and *EPSPS* gene. The results obtained from the modified CTAB method was promising, as the concentrations were higher than those in the CTAB and phenol/Chloroform methods. In addition, the purity of TSP samples was satisfactory. All the soybean samples were evidenced by presence of the *lectin* gene and *35S* promoter, *NOS* and *EPSPS* were found in all TSP samples. This is the first report showing that most of genetically modified soy protein does not use the "GMO" label in Iran, which has amplified the need for mandatory labeling systems and reliable and simple methods for routine analysis of genetically modified foods.

Keywords DNA extraction · GMO · GMO monitoring · PCR · Textured soy protein

1 Introduction

Plant breeding techniques have been developed to improve the agricultural practices and nutritional quality and produce genetically modified (GM) products that exhibit interesting traits such as insect resistance, herbicide tolerance, abiotic stress resistance and modifications in the nutrient composition (Ashrafi-Dehkordi et al. 2018; Tahmasebi et al. 2019). The new emphasis on GM foods is the solution to world hunger, which has led to an increase in the number of GM foods in the market. According to the International Service for the Acquisition of Agri-biotech Applications (ISAAA 2018), there were 191.7 Mha of planted GM plants in 26 countries in 2018. A total of 21 developing countries planted 54% (103.1 Mha) of the global GM hectares, while 5 industrialized countries accounted for 46% (88.6 Mha), while 43 non-growing countries formally regulated the import of GM crops. The United States planted 75 Mha of land in 2018 and remains the world's largest producer of GM crops, covering 39% of the global GM crops. Brazil ranks the second with 51.3 Mha (27% of global production).

Commercially grown GM crops remain largely restricted to the two main traits, namely insect resistance and herbicide tolerance, in four main crops: soybean, canola, cotton and maize (James 2013). GM soybeans have the highest adoption rate in the world, accounting for 50% or 95.9 Mhe of the global GM crop area. This region accounts for 78% of total global soybean production (ISAAA 2018).

Detecting GMOs in a wide variety of food and feed matrices is important to countries with mandatory GMO labeling laws (Sisea and Pamfil 2007). Moreover, before using GM crops to obtain GM feed for the livestock, countries may want to test unapproved GM varieties. Several countries including the EU, Brazil, Russia, and Japan have threshold

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levels range from 0.9 to 5% for the presence of GMO and the threshold limit of tolerance for unapproved GMOs is zero. Other countries (Canada and USA) have voluntary labelling or have no oblige legislation (Du et al. 2020; Santos et al. 2016; Holck and Pedersen 2011).

By using different technologies, such as indirect strategies (protein-based methods) or direct strategies (DNA-based methods), great progress has been made in the development of genetic analysis methods for the detection of GMOs in food and feed samples.(Fraiture et al. 2015; Griffiths et al. 2002; Rott et al. 2004). Analytical methods based on PCR technology are increasingly used for the detection of target DNA sequences in GMOs due to their versatility, sensitivity, specificity, and high throughput applications (Gryson et al. 2004; Morisset et al. 2009; Gürakan et al. 2011). PCR allows the selective amplification of specific segments of DNA in a mixture of other DNA sequences. Polymerase chain reaction is often used to detect regulatory sequences such as NOS-terminator (Nopalin synthase, terminator from *Agrobacterium* plasmid). In addition, CaMV 35S sequences (derived from cauliflower mosaic virus) are often used as a screening tool to detect GMO and other target genes in GMOs (Lipp et al. 2001; Holden et al. 2010; OVeSNá et al. 2010). Because, the CaMV 35S promoter is one of the most frequently modified elements in GMOs and important for screening detection of GMOs, a large variety research have used 35S promoter in screening detection of GMOs (Wu et al. 2014). DNA extraction is the first step in such analytical methods (Sarmadi et al. 2016). The goal of the extraction procedure is to isolate DNA of reasonable quantity, purity, integrity and quality to allow DNA amplification, which is often the most time consuming step of a DNA-based detection method. The efficiency of the DNA extraction step is critical to successful amplification, because there are many compounds that inhibit DNA amplification. For example, many complex processing steps are very difficult and may negatively affect the detection of GM organisms. These include cooking, heating, pH treatments, high pressure, physically shearing and high torque settings, polysaccharides, lipids and polyphenols or extraction chemicals such as CTAB (Coello et al. 2017; Anklam et al. 2002; Hübner et al. 1999; Saghai-Maroof et al. 1984). For example, pH and temperature influence the degradation of a cry1A (b) sequence in Bt-maize during preparation of polenta and in another research thermal stress in combination with pH affects DNA integrity (Bauer et al. 2003; Hupfer et al. 1998).

Due to high consumption, the import of GM soybeans has recently increased, making it even more urgent to monitor GM soybeans in the imported food. Therefore, this study describes the use of efficient, simple and low-cost DNA extraction methods. It also introduces a screening method for GM organisms in soy protein samples collected from supermarkets, traditional markets and grocery stores in the center of Fars province, Iran.

2 Materials and methods

2.1 Samples

Thirty samples of raw soybean and TSP were purchased from supermarkets, traditional markets and grocery stores in the center of Fars province. A type of experimental unreleased transformed soybean has been kindly provided from Institute of Biotechnology, Shiraz University, Iran and was used as a positive control.

2.2 Genomic DNA extraction and purification

Three in-house CTAB-based methods were also utilized, namely the standard cetyltrimethyl ammonium bromide (CTAB) precipitation of DNA protocol (Gryson et al. 2004) and another CTAB protocol with ethanol precipitation of DNA (Sisea and Pamfil 2007), which is referred to as 'modified CTAB' method in this study in order to distinguish it from the standard CTAB protocol. The modification included: First, 20 µl proteinase K (20 mg/ml) (Pishgam, Thehran, Iran) was added, and the tube was shaken and incubated at 65 °C for 3 h. In the next step, the tube was centrifuged for 20 min at about 16,000*g*, and the supernatant was transferred to a tube containing 500 µl chloroform (Merck, Germany). Subsequently, the tube was shaken for 30 s, before it was centrifuged for 10 min at 16,000*g* until phase separation started. Next, 500 µl of upper layer was transferred into a new tube. The above procedure was repeated three times. Finally, 0.6 volumes of isopropanol (Merck, Germany) was added; the tube was shaken and from less processed food (seed, powder incubated at −20 °C overnight. The third method used Phenol/chloroform and CTAB (Sigma Aldrich, USA) buffer (Ferrari et al. 2007) (Table 1).

2.3 Genomic DNA quantification and purity measurement

Total DNA concentration (ng DNA/µl extract) was used by measuring UV absorbance at 260 nm (Scan Drop2, analytic Jena, Germany). Each quantification was repeated three times.

Table 1 DNA extraction methods were used in this study

Methods	Starting material	Extraction buffer	Elution buffer	References
CTAB	100 mg	1000 µL buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris HCl pH 8.0)	50 µL sterile deionized water	Gryson et al. (2004)
Modified CTAB	100 mg	500 µL buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris HCl pH 8.0)	50 µL sterile deionized water	Sisea and Pamfil (2007)
Phenol/chloroform	100 mg	1100 µL buffer (2% CTAB, 1.4 M NaCL, 20 Mm EDTA, 100 Mm Tris-Hcl Ph 8.0) and 0.2% b-mercaptoethanol	50 µL sterile deionized water	Ferrari et al. (2007)

2.4 Oligonucleotide primers

The amplicons were amplified with specific oligonucleotide primers. Four pairs of PCR primers as listed in Table 2 were used. The primers of NOS, 35S, EPSPS, and Lec were used to detect NOS-terminator (nopaline synthase-terminator), CaMV35S promoter, *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) structure gene region, in enolpyruvylshikimate-3-phosphate synthase gene existed in Roundup Ready GM-soybean, and *Lectin* gene, respectively.

2.5 Detection by PCR

Amplifications by polymerase chain reaction (PCR) in thermal cycler (AB Applied Biosystems, USA) were carried out in reaction mixtures (20 µL) containing 2.5 µL of 10x PCR buffer, 2.5 µL of 0.2 mM dNTP (Takara, Japan), 3 µL 3 mM MgCl2 (Merck, Germany), 1 µL primers with 0.2 µM each, 0.5 unites of Taq DNA polymerase (CinaGene, Tehran, Iran) and 100 ng template DNA (extracted by modified CTAB method). Amplification was performed with a thermal cycler according to the following PCR step-cycle program for all primer pairs used: pre-denaturation of 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing for 45 s at 52 °C for Lec-F/Lec-R, 57 °C for GMO-F/GMO-R, 52 °C for 35S-F/35S-R, 61 °C for nos-F / nos-R, and extension at 72 °C for 40 s. A final extension at 72 °C for 7 min followed the final cycle for complete synthesis of elongated DNA molecules. The PCR products were checked by electrophoresis in a 1.0% agarose (CinaGene, Tehran, Iran) (w/v) gel.

3 Result and discussion

3.1 Genomic DNA assessment

The most common and quickest technique to determine DNA concentration and purity is to determine DNA by measuring the absorbance with a spectrophotometer. Table 3 summarizes the DNA yield and purity range obtained for all samples extracts using the three extraction methods. All the methods were capable of producing significantly different DNA yields. The data (Table 2) revealed that there were some differences in the purity of DNA extracts obtained by different methods. The modified CTAB method showed promising results, as the concentrations were higher than those found in the standard CTAB and phenol/chloroform methods. In addition, the purity of the TSP sample was very satisfactory. Different food products may require different DNA extraction protocols because DNA purity and concentration can be highly affected by various contaminants in sample matrices such as food processing, polysaccharides, lipids and polyphenols or physical parameters and extraction

Table 2 The list of primer pairs used in this study

Name	Target	5′- 3′ sequence	Annealing temp. (°C)	Amplicon (bp)	References
35SF-1	P-35S	GCTCCTACAAATGCCATCA	52	195	Cardarelli et al. (2005)
35SR-1		GATAGTGGGATTGTGCGTCA			
nos-F	T-NOS	GCATGACGTTATTTATGAGATGGG	61	118	Cardarelli et al. (2005)
nos-R		GACACCGCGCGCGATAATTTATCC			
Lec-F	Lectin gene	AGCTGGAACAAGTTCGTGC	52	343	Gryson et al. (2004)
Lec-R		CGACTTGATCACCAGACTCG			
GMO-F	*EPSPS* gene	ATCCCACTATCCTTCGCAAGA	57	169	Cardarelli et al. (2005)
GMO-R		TGGGGTTTATGGAAATTGGAA			

Table 3 Summary of DNA yield and purity for TSP samples using different DNA extraction methods

DNA extraction methods	DNA yield (ng / µl) Mean ± SD	DNA purity A260 nm/ A280 nm ratio	A260 nm/ A230 nm ratio
Modified CTAB	296.66 ± 64.29	1.81 ± 0.07	1.86 ± 0.11
CTAB	213.33 ± 37.85	1.52 ± 0.04	1.85 ± 0.12
Phenol/chloroform	126.66 ± 30.55	1.86 ± 0.02	1.53 ± 0.05

chemicals such as CTAB (Anklam et al. 2002; Xia et al. 2019). For example the CTAB method was suitable for extracting DNA from complex foodstuffs and difficult samples and the SDS-based method was suitable for extracting DNA from less processed food (seed, powder, meal) (Wang et al. 2016; Elsanhoty et al. 2011; Peano et al. 2004). Moreover, the degraded DNA could be explained by the type of processing in the product elaboration, which decrease the average fragment length of the extracted DNA (Nikolić et al. 2017; Toyota et al. 2006). For example, one can use acidic or alkaline reagents that can cause DNA hydrolysis and degradation or prolonged exposure to high temperatures and the successive steps of ethanol extraction, leading to DNA fragmentation, which affects the visibility of DNA in agarose gel and PCR analysis (Du et al. 2020; Coello et al. 2017). Therefore, the concentration of DNA extracted from the samples are low and fragmented, and the DNA cannot be seen in the agarose gel (Fig. 1).

In this study we employed a three step analysis to determine the identity of GMO samples. The first step involved genomic DNA extraction and the amplification of specific soy sequence (*lectin* gene) from TSP DNA, necessary to discriminate between negative and positive results due to inhibition in the amplification (Forte et al. 2005; Nikolić et al. 2008). The second step required the amplification of GMO-specific sequence, represented by the 35S promoter and NOS terminator (the most common recombinant elements in GM crops), to screen for the presence of transgenic material in the samples (Fraiture et al. 2016; Safaei et al. 2019). These genetic control elements are present in around 95% of currently commercialized GM crops in EU (Forte et al. 2005). Barbau-Piednoir et al. (2010) reported 17 out of 24 EU-authorized GMOs contain either, 15 out of 24 contain the 35S promoter, the 15 out of 24 EU-authorized GMOs contain NOS terminator or 9 out of 24 EU-authorized GMOs contain both. In another study, Wu et al. (2014) reported that 67 out of 102 (65.7%) of approved commercial GM events contain the 35S promoter, 55 out of 102 (53.49%) the NOS terminator, and 83 out of 102 (81.4) either one or both in their transgene constructs. Then, GMO-containing samples were subjected to analysis of specific transgenic material

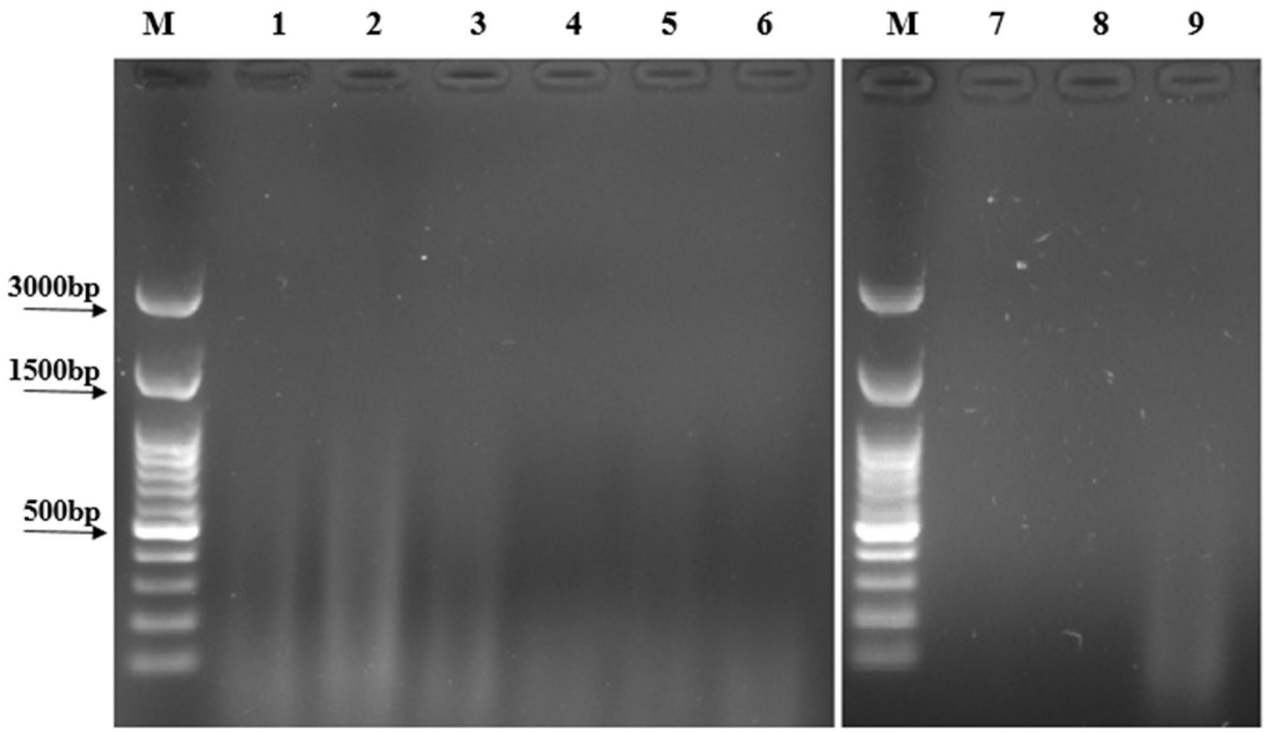

Fig. 1 Agarose gel electrophoresis of genomic DNA using modified CTAB (lanes 1, 2, 3), CTAB (lanes 4, 5, 6), phenol/chloroform (lanes 7, 8, 9) methods and M, 100 bp DNA ladder (SMOBIO, Taiwan, DM2300)

(Roundup Ready Soy specific gene) to determine the type of GMO present (Lin et al. 2000).

Thirty samples were analyzed. The results showed that all TSP samples were positive for the three introduced genetic elements, the promoter (*35S*), terminator (*NOS*) and GMO genes. Agarose gel electrophoresis of the PCR amplified products from the samples resolved a band of approximately 343 bp for the detection of *lectin*, a band of 169 bp for GMO, a band of 118 bp for NOS, and a band of 195 bp for P35S (Fig. 2). Non-transgenic soy was used as the negative control. All of the positive samples in this study came from unclear source and domestic processed products (supermarket and traditional market). By using *lectin* gene primers for soybean endogenous genes, the test sample was also configured as a soybean product. Several reports have been published describing PCR techniques used to detect GM soybeans (Lin 2001; Lin et al. 2000). For example, Sarmadi et al. (2016) reported that 3 out of 5 imported soybean examined were found to contain the *35S*-promoter, *NOS*-terminator and *EPSPS* genes. Likewise, Lin et al. (2001) reported that out of the 28 commercial GM crops examined, 22 contained 35S promoter or NOS terminator genes. Safaei et al. (2019) reported 2 out of 81 rice samples tested were positive for *35S*-promoter but not positive for *NOS*-terminator. They also mentioned that conventional PCR was capable to identify the GMO. In another study, Oraby et al. (2005) reported that 12.5% of the food product tested were positive for *35S*-promoter, while they were negative for *NOS*-terminator.

4 Conclusion

The present study contributes to the accumulation of basic data necessary to consider the impact of food processing on DNA based detection. Three methods of DNA extraction were used to extract DNA from textured soy protein. The "modified CTAB" yielded satisfactory results, as concentrations were higher than those in the standard CTAB and phenol/chloroform methods, and the purity of TSP was satisfactory. In addition, the "modified CTAB" was a cost-effective method.

In short, PCR method was used to monitor products derived from GMO, which were sold in Shiraz markets, Iran. All samples were evidenced by presence of the *lectin* gene and *35S* promoter, *NOS* and *EPSPS* were found in all TSP samples. These results showed that all samples purchased randomly from the markets in Shiraz were

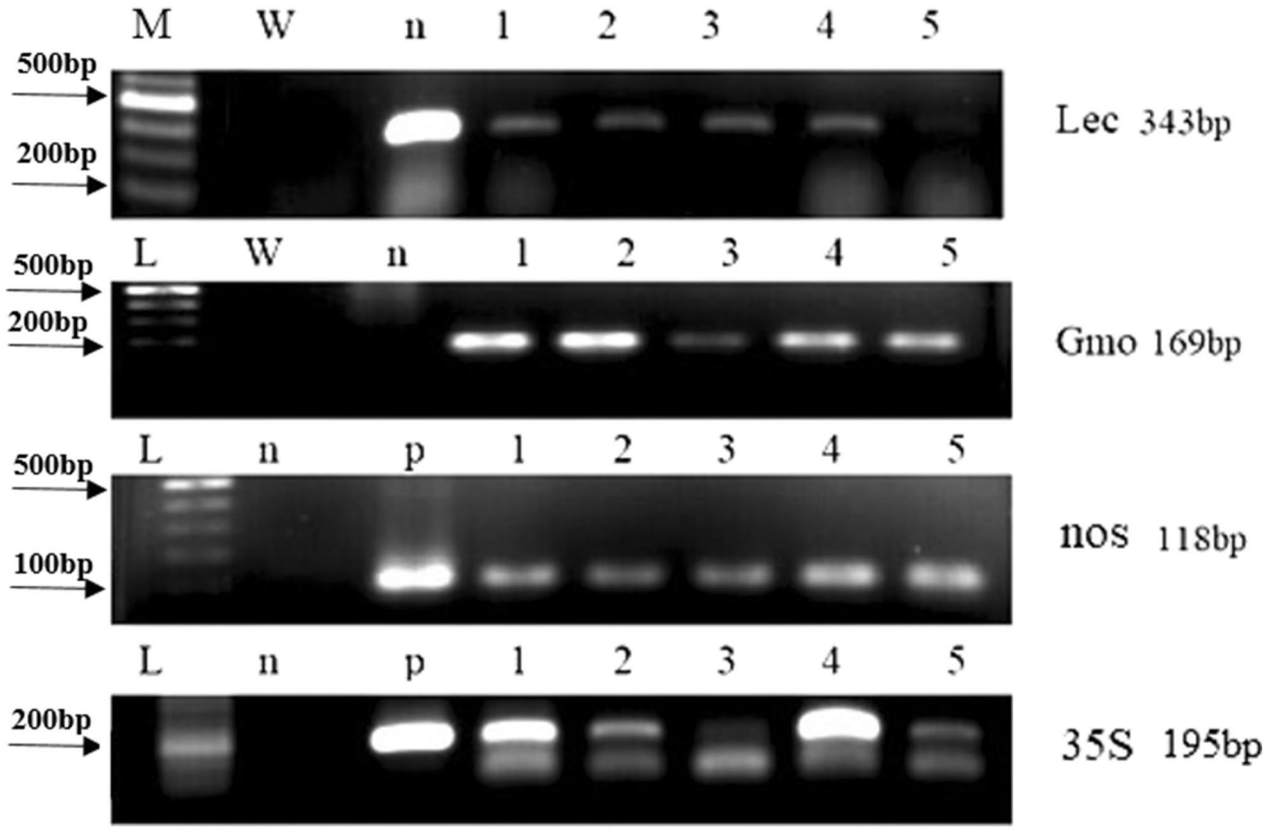

Fig. 2 Representative agarose gel electrophoresis of PCR products. Lanes: 1, water (negative control, without DNA); n, non-GMO; 1–5, GMO; P (positive control) and M, 100 bp DNA ladder

transgenic. Because most of the soybeans consumed in Iran are imported from the countries that practise GM soybeans cultivation, the detection and quantitative analysis of GM soybeans in raw soybeans and processed food/feed is one of the most important requirements to consumers for food safety and quality.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no competing interests associated with the manuscript.

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