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PCR-based investigation on transgenic maize status in corn processed products

Original Article

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Abstract:

Monitoring genetically modified organisms (GMOs) is increasingly required by law in countries with GMO labeling regulations. Efficient extraction of high-quality genomic DNA is essential for detecting and quantifying GM content in foods using PCR techniques. This study developed and evaluated various DNA extraction methods from corn seeds and products to ensure sufficient DNA yield free of contaminants and PCR inhibitors. Thirty samples, including corn seeds and derived products, were randomly purchased from supermarkets and grocery stores. Genomic DNA was extracted by different methods, and PCR was used to detect GMO-specific regions: NOS-terminator, 35S promoter, and EPSPS. The *zein* gene was also amplified to confirm the presence of the maize genome in all samples. Results indicated that 14 samples contained the NOS-terminator, 13 the 35S promoter, and one the EPSPS region, identifying 17 (57%) samples as transgenic. The methods proved effective for extracting quality DNA suitable for GMO detection. Many GM-positive products lacked labeling, underscoring the need for reliable detection methods and stricter labeling enforcement.

Key words:
Genetically modified organism, labeling, Maize, PCR

Apstrakt:

Ispitivanje prisustva transgenog kukuruza u prerađenim proizvodima od kukuruza zasnovano na PCR metodi

Praćenje genetički modifikovanih organizama (GMO) sve je češće zakonski zahtevano u zemljama koje imaju propise o označavanju GMO proizvoda. Efikasna ekstrakcija genomske DNK visokog kvaliteta od suštinskog je značaja za detekciju i kvantifikaciju GMO sadržaja u hrani primenom PCR tehnika. U ovom radu razvijene su i procenjene različite metode ekstrakcije DNK iz zrna kukuruza i proizvoda od kukuruza, kako bi se obezbedio dovoljan prinos DNK bez prisustva kontaminenata i inhibitora PCR reakcije. Trideset uzoraka, uključujući zrna kukuruza i prerađene proizvode, nasumično je nabavljeno iz supermarketa i prodavnica. Genomska DNK je ekstrahovana različitim protokolima, a PCR je korišćen za detekciju GMO-specifičnih regiona: NOS terminator, 35S promotor i EPSPS. Gen *zein* je amplifikovan radi potvrde prisustva genoma kukuruza u svim uzorcima. Rezultati su pokazali da je 14 uzoraka sadržalo NOS terminator, 13 uzoraka 35S promotor, a jedan uzorak EPSPS region, čime je ukupno 17 uzoraka (57%) identifikovano kao transgeno. Primenjene metode pokazale su se efikasnim za ekstrakciju DNK visokog kvaliteta pogodnu za detekciju GMO. Značajan broj GMO-pozitivnih proizvoda nije imao odgovarajuću oznaku, što ukazuje na potrebu za pouzdanim metodama detekcije i strožom kontrolom označavanja.

Ključne reči:
genetički modifikovani organizam, označavanje, kukuruz, PCR

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Introduction

Recombinant DNA (rDNA) technologies have been developed to improve modern farming and have provided many advantages in crops such as herbicide tolerance, biotic and abiotic stress resistance, and improved nutritional value (Ashrafi Dehkordi et al., 2021; Dehkordi et al., 2018). Many GMCs (Genetically Modified crops) have been approved since the mid-1990s worldwide, and the global area of GMCs was more than 190 million hectares in 2019 (ISAAA, 2019) and is expected to continue to increase. More than 200 different GMCs with varying features, of which the highest cultivated

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area belongs to soy, corn, canola, and cotton, and several products of these GMCs are available in markets (Ashrafi-Dehkordi et al., 2022).

Maize is one of the major agricultural crops and the cultivation of GM maize is increasing worldwide. The global area under GM maize is more than 60 million hectares, accounting for about 32% of global corn production (ISAAA, 2019). Although, maize is approved for use as feed/food in many countries based on their safety evaluation criteria. However, many consumers are demanding information and appropriate labeling for foods derived from GMOs. In this regard, different labeling systems for GM foods have been introduced in different countries such as the European Union (EU), Korea, Russia, and Japan. There are different thresholds (between 0 and 5%) for accidental mixing of GM crops; for example, the thresholds in Russia are 0.9%, in the European Union are 0.9%, in Korea, 3% and 5% in Japan (Chiaravutthi et al., 2022). Different technologies are used for the detection of GMOs in food samples, such as protein-based and DNA-based methods (Fraiture et al., 2015; Griffiths et al., 2002; Rott et al., 2004). PCR-based methods such as conventional PCR, multiplex PCR, and RT-PCR methods have become the primarily used approaches for GMO detection, which can detect even small amounts of the novel DNA in both raw and processed foods, most widely used, because of its sensitivity, specificity, versatility, and high throughput application (Li et al., 2022; Zhang et al., 2013).

On the other hand, food processing can affect the quality and quantity of DNA, leading to its removal or degradation. Moreover, for the successful DNA amplification step, one must have an efficient DNA extraction step, because there are different compounds that inhibit amplification of DNA, such as lipids, polysaccharides, and polyphenols, or extraction chemicals such as phenol, CTAB, and isopropanol (Anklam et al., 2002; Hübner et al., 1999; Saghai-Maroof et al., 1984). Therefore, in order to be able to amplify DNA, it is necessary to use an extraction method that provides DNA with an acceptable quantity, quality, and purity. This step is often very time-consuming. To identify transgenic organisms, an endogenous reference gene is needed as a standard to confirm species identification and determine the quality of genomic DNA. Various studies have been conducted to confirm internal control genes for different plants. For example, the *zein*, *zSSIIb*, and *invertase* genes in corn (Germini et al., 2004; Hernández et al., 2004; Pan et al., 2006), Lectin in soybean (Terry et al., 2001), *SPS*, *oryzain beta*, and *ppi-PPF* in rice (Chaouachi et al., 2007; Hernández et al., 2005), and *β-fructosidase* in the Solanaceae family (Chaouachi et al., 2008). The elements such as the CaMV35S promoter (CaMV), nopaline synthase (NOS) terminator, and selectable markers including the *pat/bar* gene, *HPT* gene, and the *NPTII* gene are commonly derived from naturally occurring viruses, bacteria, and fungi found in many GMOs (Li et al., 2022). These elements are usually used for detection targets in GMO screening. This screening is a rapid and cost-efficient method for the detection of samples potentially containing one or several GMOs.

In this study, three DNA extraction methods were employed for isolating DNA from maize samples, and a PCR method was used to detect GM maize in various processed foods collected from the Shiraz market.

Materials and Methods

Samples collection

Thirty samples, including raw corn and its products (corn puff, corn nut, canned corn, corn starch, corn flour, and popcorn), were bought from supermarkets in Shiraz. **Tab. 1** provides an overview of the corn samples collected from supermarkets in Shiraz. The table lists each sample's identification number, product name, and product type, offering a clear classification of the various corn-based foods analyzed in this study.

DNA extraction

Three different CTAB-based (cetyltrimethyl ammonium bromide) methods were used for DNA extraction from the samples. The first method, referred to as "Method 1," follows the standard CTAB precipitation of DNA protocol (Gryson et al., 2004) and was applied to raw corn, corn nut, and corn flour. The second method, referred to as "Method 2," uses the CTAB protocol with ethanol precipitation of DNA (Sisea & Pamfil, 2007) and was applied to canned corn, corn puff, and popcorn. The third method, referred to as "Method 3," follows another CTAB protocol (Waiblinger et al., 2012) and was used for corn starch.

Method 1: The DNA samples were extracted from raw corn, corn nuts, and corn flour using the standard CTAB method. Following grinding samples with a mortar and pestle, the powder was mixed with 1000 µL of buffer (containing 100 mM Tris-HCl, 20 mM Na_2EDTA, 1.4 M NaCl, and 20 g/L CTAB, pH 8.0). The sample mixtures were transferred to 2-ml tubes and were incubated at 65 °C for 90 minutes in a water bath. The mixtures were gently rotated every 10 minutes for intermittent mixing before being centrifuged for 20 minutes at 13,000g. The resulting supernatant was transferred to a fresh 2-ml tube, followed by the addition of 500 µL of chloroform (Merck, Germany) and vortexing for 30 seconds.

The tube was centrifuged again at 13,000 g for another 20 minutes. The upper phase was transferred to a fresh tube containing 0.7 volumes of pre-chilled isopropanol (Merck, Germany) and kept overnight at -20 °C. The mixture was centrifuged at 13,000 g for 10 minutes. The supernatant was discarded, and the pellet was washed with 500 µL of 70% ethanol (Merck, Germany). After that, the pellet was subjected to centrifugation for 10 minutes at 13,000 g. After the removal of the supernatant, the pellet was air-dried. Subsequently, the DNA was dissolved in 50 µL SDW.

Method 2: The DNA sample was extracted from canned corn, corn puffs, and popcorn using the modified CTAB method. The samples were ground using a mortar and pestle. Before milling, liquid nitrogen was added to the popcorn, and the canned corn was heated to below 40 °C. Then, the powder was mixed with 1000 µL of buffer (containing 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na2EDTA, 20 g/L CTAB, and pH 8.0) in a 2-mL tube, with the addition of 25 µL of 1% (w/v) sodium dodecyl sulfate (SDS) to the corn puffs and popcorns. The mixture was incubated in a water bath at 65 °C for 2-4 hours. The tubes were gently inverted every 10 minutes for intermittent mixing. After that, 20 µL proteinase K (20 mg/L) was added and incubated at 37 °C for 45 minutes. Next, the tube was centrifuged at 13,000 g for 20 minutes. The supernatant was transferred to a new 2-mL tube containing 500 µL of chloroform (Merck, Germany) and was shaken for 30 seconds. The tube was centrifuged for 20 minutes at 13,000 g. The upper phase was transferred to a new tube, and two volumes of CTAB precipitation buffer (5 g/L CTAB and 0.04 M NaCl) were added, and the tube was incubated at room temperature for 60 minutes. Then the tube was centrifuged for 20 minutes at 13,000 g. The supernatant was discarded, and the precipitate was dissolved in 350 µL chloroform and 350 µL1.2 M NaCl. The mixture was shaken for 30 seconds, and the tube was centrifuged for 20 minutes at 13,000 g. The upper phase was transferred to a new tube. The 0.7 volumes of pre-chilled isopropanol were added, and the tube was shaken and kept overnight at -20 °C. The tube was centrifuged for 20 minutes at 13,000 g. The supernatant was discarded and washed with 500 µL pre-chilled ethanol (70%) (Merck, Germany). The tube was shaken carefully, and then centrifugation was done for 20 minutes at 13,000 g. The pellet was air-dried, and DNA was dissolved in 50 µL SDW.

Method 3: The DNA sample was extracted from corn starch using the modified protocol of Waiblinger et al. (2012). The sample (5 g) was mixed with 25 mL of CTAB buffer (0.1 M Tris-HCl, 1.4 M NaCl, 20 mM Na_2EDTA, and 20 g/L CTAB, pH 8.0), and then 50 µL of α-amylase solution (10 mg/mL) was added to the mixture. The sample mixture was transferred to a 50-mL tube and incubated at 65 °C for 4 hours in a water bath, with the tubes gently inverted every 10 minutes for intermittent mixing. The tube was centrifuged for 20 minutes at 13,000 g. The supernatant was transferred to a new 50-mL tube containing 10 mL of chloroform (Merck, Germany)

Table 1. Sample code, names of corn samples and product types collected from supermarkets in Shiraz

Sample code	Product name	Product type
1	Corn seed	Nut
2	Corn seed	Nut
3	Corn seed	Nut
4	Corn seed	Nut
5	Corn seed	Nut
6	Corn seed	Nut
7	Corn seed	Nut
8	Corn seed	Nut
9	Corn seed	Nut
10	Corn seed	Nut
11	Bulk corn seed	Nut
12	Bulk corn seed	Nut
13	Bulk corn seed	Nut
14	Canned corn	Canned
15	Canned corn	Canned
16	Canned corn	Canned
17	Canned corn	Canned
18	Raw seed	Raw corn
19	Raw seed	Raw corn
20	Raw seed	Raw corn
21	Corn flour	Flour
22	Corn flour	Flour
23	Popcorn	Pop corn
24	Popcorn	Pop corn
25	Popcorn	Pop corn
26	Extruded snack	Puff
27	Extruded snack	Puff
28	Extruded snack	Puff
29	Corn Starch	Starch
30	Corn Starch	Starch

Table 2. The list of primer pairs used in this study

Name	5'-3' sequence	Target	Amplicon (bp)	Refrence
Zm-F	CGCCAGAAATCGTTTTTCAT	*Zein*	139	(Germini et al. 2004)
Zm-R	GGTGGTGTCCTTGCTTCCTA	*Zein*	139	(Germini et al. 2004)
35SF-1	GCTCCTACAAATGCCATCA	P-35S	195	(Cardarelli et al. 2005)
35SR-1	GATAGTGGGATTGTGCGTCA			
nos-F	GACACCGCGCGCGATAATTTATCC	T-NOS	118	(Cardarelli et al. 2005)
nos-R	GACACCGCGCGCGATAATTTATCC			
GMO-F	ATCCCACTATCCTTCGCAAGA	*EPSPS*	169	(Cardarelli et al. 2005)
GMO-R	TGGGGTTTATGGAAATTGGAA			

and was shaken for 30 s. The tube was centrifuged at 13,000 g for 20 minutes. The upper phase was transferred to a new tube with 0.7 volume of pre-chilled isopropanol (Merck, Germany) and then incubated overnight at -20 °C. The centrifugation was done at 13,000 g for 10 minutes. The supernatant was discarded, and the pellet was transferred to a 2-mL tube and washed with 1500 µL70% ethanol (Merck, Germany). The centrifugation was performed at 13,000 g for 10 minutes. Finally, the supernatant was discarded, and the pellet was air-dried. The DNA was dissolved in 50 µL SDW.

DNA quantification and purity measurement

DNA concentration was measured by an Eppendorf spectrophotometer (ScanDrop2, Analytic Jena, Germany), using the UV absorbance at 260 nm (nanograms of DNA per microliter extract).

Oligonucleotide primers

Specific oligonucleotide primers were used for amplifying amplicons. The primers of NOS, 35S, EPSPS, and Zm were used to detect NOS-terminator, CaMV35S promoter, *EPSPS* structure gene region, and *Zein* gene (as an endogenous control), respectively. The sequences of oligonucleotide primers are given in **Tab. 2**.

Table 3. Reaction conditions for *Zein*, CaMV 35S, NOS and *EPSPS* amplification

Step	*Zein*	CaMV 35S	NOS	*EPSPS*
Initial denaturation	95 °C, 3 min	95 °C, 3 min	95 °C, 3 min	95 °C, 3 min
Denaturation	95 °C, 45S	95 °C, 45S	95 °C, 45S	95 °C, 45S
Annealing	50 °C, 45S	52 °C, 45S	61 °C, 45S	57 °C, 45S
Extension	72 °C, 40S	72 °C, 40S	72 °C, 40S	72 °C, 40S
Final extension	72 °C, 10 min	72 °C, 10 min	72 °C, 10 min	72 °C, 10 min
Cycles	35	35	35	35

Polymerase chain reaction

PCR in thermal cycler (AB Applied Biosystems, Veriti 96 well, USA) was used in reaction mixtures (20 µL) containing 2.5 µL of 4 mM dNTP, 2.5 µL of 10 x PCR buffer, 3 µL DNA, 1.5 µL 25 mM $MgCl_2$, 0.6 µL primers with 20 µM each and 5 unites/µL of Taq DNA polymerase (Sinagene, Tehran, Iran). A thermal cycler was used for amplification according to the following PCR step-cycle program for all primers, as shown in **Tab. 3**. The analysis of PCR products was done on a 1.0% agarose gel electrophoresis.

Results

Three different CTAB-based DNA extraction methods were applied to the corn and corn-derived food samples presented in **Tab. 1**. A summary of the DNA yields and purity ranges obtained using the three extraction methods is presented in **Tab. 4**. The results show that there are discrepancies in the DNA extraction between the three methods. Following the genomic DNA extraction, the PCR method was used to identify the GMO samples.

Firstly, the specific maize sequence, the *zein* gene, was employed to distinguish negative and positive outcomes resulting from amplification interference (Forte et al., 2005). Secondly, for the identification of GM material in corn samples, the 35S-promoter and NOS-terminator were employed. Subsequently, the samples were analyzed to identify the specific type of GMO by amplifying the Roundup Ready gene using specific primers (Lin et al., 2000). The electrophoresis results of the PCR products showed that the *zein* gene (a band of about 137 bp) of all corn samples was successfully amplified. This result showed that the

Table 4. Summary of Mean ±SD, DNA yield and purity for corn samples using different DNA extraction methods

DNA extraction methods	DNA yield (ng/µL)	DNA purity	
		A260nm/A280nm ratio	A260nm/A230nm ratio
Method 1	123.66±39.84	1.77±0.33	1.87±0.11
Method 2	122±69.70	1.89±0.47	1.67±0.13
Method 3	85.49±4.90	1.95±0.04	1.78±0.03

isolated DNAs have good quality and contain maize DNA (**Fig. 1**).

The positive detection of targetp genes in each individual corn sample is summarized in **Tab. 5**. This table presents the sample numbers alongside the specific target detected through PCR analysis, providing a clear and detailed overview of GMO presence in the analyzed samples. Electrophoresis of the PCR products showed a 118-bp band for NOS (**Fig. 2A**), a 195-bp band for P35S (**Fig. 2B**), and a 169-bp band for the EPSPS gene (**Fig. 2C**). The results showed that 13, 14, and one sample were positive for the 35S, NOS, and *EPSPS* gene, respectively.

Discussion

The quantity and integrity of DNA depend on the extraction method and the type of food. Contaminants present in sample matrices, including lipids, polysaccharides, polyphenols, and extraction substances, can affect DNA purity (Anklam et al., 2002). The food processing techniques, such as enzymatic activities, low pH, and heat, affect the integrity and quantity of DNA, so extracting DNA in sufficient purity and quantity is a difficult step (Peano et al., 2004; Vijayakumar et al., 2009). The different methods of DNA extraction applied in this study are capable of extracting sufficient amounts and quality DNA to identify the GM corn. A more detailed comparison of our results with previous studies further illustrates the role of extraction methods and sample matrices in DNA yield and integrity. In this study, three CTAB-based DNA extraction methods were applied to various corn and corn-derived food samples. The results showed noticeable differences in both DNA yield and purity between the methods. As shown in **Tab. 4**, Method 1 produced the highest DNA yield (123.66±39.84 ng/µL) but with slightly lower purity ratios (A260/280=1.77±0.33; A260/230=1.87±0.11) compared to Method 3, which yielded lower DNA concentration (85.49±4.90 ng/µL) but the highest purity (A260/280=1.95±0.04; A260/230=1.780±0.03). These findings suggest that Method 1 is more effective in quantity, while Method 3 provides purer DNA, possibly due to better removal of PCR inhibitors.

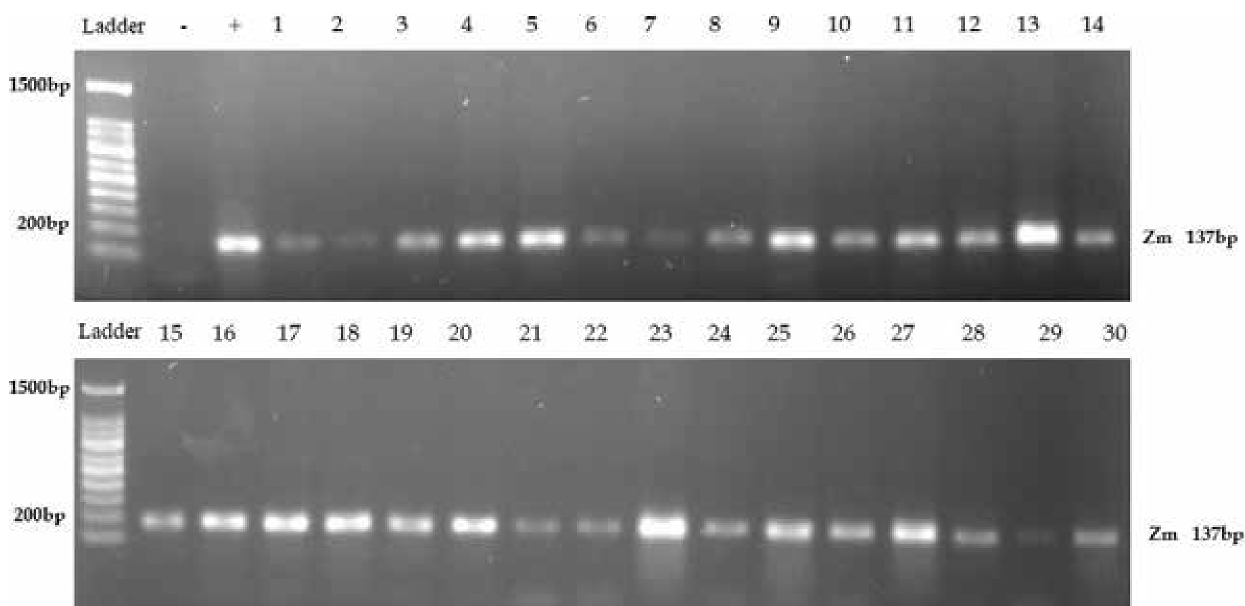

Fig. 1. Representative agarose gel electrophoresis of maize PCR products (137 bp for the detection of *zein* gene): Ladder: 100 bp DNA ladder, -: negative control,+: positive control, Lane 1-13: corn nut, Lanes 14-17: canned corn, Lane 18-20: raw corn, Lane 21-22: corn flour, Lane 23-25: popcorn, Lane 26-28: corn puff, Lane 29-30: corn starch.

Table 5. Summary of positive PCR detection of target in individual corn samples

Sample code	Pro1	nos	GMO
1	×	✓	×
2	✓	×	×
3	×	×	×
4	×	×	×
5	×	×	×
6	×	×	×
7	×	✓	×
8	✓	✓	×
9	×	×	×
10	×	✓	×
11	×	✓	×
12	×	×	×
13	✓	×	×
14	✓	✓	✓
15	✓	✓	×
16	✓	×	×
17	✓	✓	×
18	✓	✓	×
19	✓	✓	×
20	✓	✓	×
21	✓	✓	×
22	✓	✓	×
23	×	×	×
24	×	×	×
25	×	×	×
26	×	×	×
27	×	×	×
28	✓	✓	×
29	×	×	×
30	×	×	×

These results are consistent with findings by Peano et al. (2004), who reported that DNA yield and integrity varied widely depending on the extraction method and the complexity of the food matrix. In their study, the NucleoSpin Food kit was most effective in extracting DNA from complex matrices, while Wizard and QIAamp kits performed better for simple matrices like flours. Similarly, Vijayakumar et al. (2009) demonstrated that intense food processing, such as autoclaving and baking, significantly reduces DNA fragment size, resulting in the complete degradation of long DNA fragments and the retention of only short fragments (<500 bp). Nevertheless, successful PCR amplification of short amplicons was still possible, highlighting the importance of using appropriate primer design and extraction protocols for reliable GMO detection in processed foods.

In this study, PCR results using specific primers showed that 17 (57%) samples were transgenic. PCR techniques have been widely employed in several studies to detect genetically modified (GM) foods. For instance, Lin et al. (2001) reported that the 35S or NOS were present in 22 out of 28 commercial genetically modified crops. Fifty samples of meat products processed in Serbia were tested using the PCR method. The results showed that 12 out of the 50 samples contained GM soybeans (Taski-Ajdukovic et al., 2009). Results from another study showed that 60 of 66 food samples contained RR soy (Cardarelli et al., 2005). In another study, 100 food samples were randomly collected from Turkish markets, and transgenic soybeans and corn were identified using PCR; 25% of the samples were transgenic (Arun et al., 2013). Ujhelyi et al. (2008) reported that 38% of random samples collected in Hungary were transgenic and that 7 out of 80 foods contained RR soy. However, they did not contain the NOS terminator. Gurakan et al. (2011) reported that the 35S promoter was present in 11 out of 31 commercial Turkish food products containing corn.

Conclusion

In this study, three simple and cost-effective methods were used to extract DNA from maize seeds and their products. Subsequently, PCR was employed to screen for genetically modified organisms (GMOs)-derived products. For screening products derived from GMOs, the PCR method was used. The isolated DNA of all samples was confirmed by the presence of the *zein* gene. The 35S promoter, NOS, and *EPSPS* were found in a number of samples. These results show that 17 samples (57%) are transgenic, which is consistent with the fact that Iran imports the majority of its corn from countries cultivating genetically modified corn. Therefore, the detection and labeling of these products is one of the demands of consumers regarding food safety and quality.

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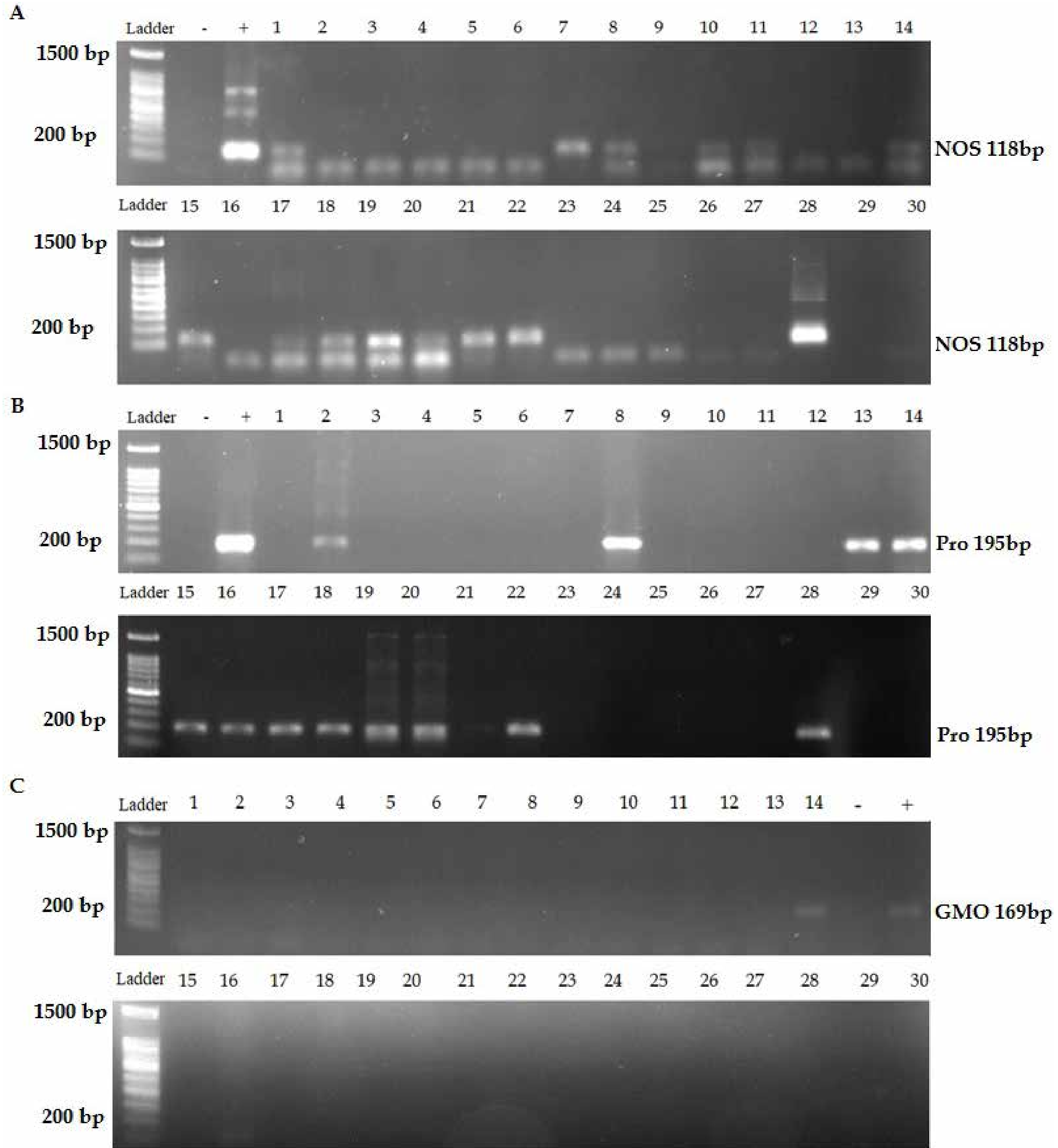

Fig. 2. Representative agarose gel electrophoresis of maize PCR products: Ladder: 100 bp DNA ladder, -: negative control,+: positive control, Lane 1-13: corn nut, Lanes 14-17: canned corn, Lane 18-20: raw corn, Lane 21-22: corn flour, Lane 23-25: popcorn, Lane 26-28: corn puff, Lane 29-30: corn starch. A band of 118 bp for NOS (**Fig. 2A**), a band of 195 bp for P35S (**Fig. 2B**), and a band of 169 bp for the *EPSPS* gene (**Fig. 2C**)

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