Journal of Food Research; Vol. 9, No. 2; 2020
ISSN 1927-0887 E-ISSN 1927-0895
Published by Canadian Center of Science and Education

Evaluation of a Real Time PCR Assay Method for the Detection of Genetically Modified Organisms in Food Products

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Received: April 2, 2019 Accepted: January 3, 2020 Online Published: February 15, 2020
doi:10.5539/jfr.v9n2p1 URL: https://doi.org/10.5539/jfr.v9n2p1

Abstract

The objective of the study was to determine qualitatively by validated Real Time PCR method the occurrence of genetically modified maize and soybean in commercial food products from the Greek market. 70 independent samples were collected, including products from different categories (i.e. cereal based, biscuits and snacks) which declared either corn or soybean on the labelling. The result of the study indicated that 37.1% of maize and soy products (n=70) displayed in the Greek market have detectable levels of genetically modified maize or soy. These products were identified by specific primers and included common GMO detection primers for 35S and NOS terminator. Adequate repeatability and reproducibility was demonstrated for the applied Real Time PCR method, as evaluated by intra- and inter-laboratory tests.

Keywords: Reat-Time PCR, GMOs, maize, soybean, 35s promoter, NOS terminator, lectin, zein

1. Introduction

1.1 Genetically Modified Organisms in Food Industry

The use of genetically modified organisms (GMOs) as foods and food products is becoming more and more widespread. Since the official GMO approval in 1996, adoption of GM technology has been growing exponentially and the number of GM crops introduced to the global market has been increasing. Although the vast amount of GM crops is cultivated outside Europe, the EU is nevertheless faced with the consequent introduction of GM to its market and should respond as foreseen by its legislation (Rosa et al., 2016). Thus, the EU has established a strict regulatory framework to trace GMOs in foods and food ingredients from production to the point of consumption. Once a food product has been found to be positive for one or more GM events (i.e. Roundup Ready® soybean, Bt-176 maize, Bt-11 maize, MON810 maize, and T25 maize), the subsequent analytical steps consist of assessing compliance with the current legislation (EC 1829/2003, EC 1830/2003) and declare labelling as containing GMOs.

1.2 Explore Importance of the Problem

The surveillance of food labelling with respect to GMOs requires DNA-based analytical techniques. The methods which can be used to detect GMOs are the polymerase chain reaction (PCR), protein based methods and phenotypic characterization. Among those methods, PCR method is the only one which can provide a general screening for GM varieties. The polymerase chain reaction (PCR) is highly specific and sensitive method for the detection of nucleic acids (DNA or RNA) and a vast number of PCR systems for food control exists, as for example for the detection of pathogenic microorganisms or of food components as well as for the identification of animal species in meat products. PCR also provides a reliable tool for the identification of GMO containing food in order to control food labeling regulations concerning GMOs. Several analytical methods using PCR

technology have been developed to qualitatively detect or quantitatively determine the presence of a modified sequence of nucleic acid in transgenic food (Gachet, Martin, Vigneau & Meyer, 1998; Rosa et al., 2016; Wang et al., 2019). A quantitative PCR procedure, which applies the ABI Prism 7700 sequence detection system has been proposed as a more accurate and less labor-intensive method. This procedure is known as TaqMan (Holland et al., 1991) and is based on the application of a fluorogenic probe that hybridizes within the target sequence bound by usual PCR primers (Vaïtilingom, Pijnenburg, Gendre & Bringnon, 1999). The efficiency and operation of the conventional DNA-based assays are still low and are often not adequate for a timely in field testing (Qian, Wang, Wu, Ping, & Wu, 2018).

1.3 Evaluation of a PCR Method for Detection of GMOs

In the present study, a molecular screening method based on Real Time PCR that involves amplification of specific soya or maize sequences from plant DNA and the amplification of 35S promoter and NOS terminator for the detection of GM soya and maize was developed.

2. Materials and Methods

2.1 Food Products

A total of 70 samples of widely consumed products, namely 40 cereal based products, 10 biscuits, and 20 snacks were collected from the Greek market during the period of September 2018 to February 2019. The samples were separated in 2 categories according to the declarations on their packaging. The first category (category I) included 40 products that were labeled as "it contains corn' (25 cereal based products, and 15 snacks) while the second category (category II) included 30 products which bared the indication "it contains soya" (15 cereal based products, 10 biscuits and 5 snacks).

Standard curves were calibrated with commercial transgenic soybean and maize reference standards (Fluka, Buchs, Switzerland). These standards are Certified Reference Material consisting of dried soybean and maize powders and were developed by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) for the European interlaboratory trial mentioned above.

2.2 Genomic DNA Extraction and Quantification

The suitability of isolated DNA as an analyte for PCR-based detection of GMOs will depend on the quality, purity, and quantity of the DNA. The purity of extracted DNA can be assessed by measurement of A_{260}/A_{280} and A_{260}/A_{230} UV absorption ratios with a spectrophotometer. When the 260/280 nm absorption ratio is between 1.5 and 2.0 and the 260/230 nm absorption ratio is more than 1.7 the extracted DNA should be suitable for PCR analysis.

DNA isolation was implemented using the following procedure. NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Germany) USA) was used for the DNA extraction of all reference standards and foods. The extraction method was applied according to the manufacturer's instructions with some modifications. Approximately 100 mg of each sample were used for the extraction, after grinding in liquid nitrogen. The sample was incubated with the Lysis Buffer and the Proteinase-K overnight at 65°C. After the lysis, the precipitation with absolute Ethanol and the washing steps DNA was eluted in duplicate in order to increase the concentration. DNA concentration was determined spectrophotometrically. All samples were tested neat and diluted 10^{-1} in dH_2O.

2.3 Real Time PCR

2.3.1 Real Time PCR Assay for Corn and Soya

The protocol was an in-house established Real Time-PCR assay using the primers of (Brodmann et al. 2002, Studer et al. 1998). Real Time PCR targets were the lectin and zein genes amplifying the 414 bp and 217bp fragments, respectively. Reactions were performed in a 25 μL final volume, containing 12.5 μL of Master Mix (KAPA SYBR GREEN Fast qPCR, KAPA BIOSYSTEMS), 0.9 μM of each primer and 7.5 μL of eluted DNA to make up for 25 μL. Amplification conditions consisted of a 10 min initial denaturation step at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 60 s annealing and elongation at 60°C.

2.3.2 Real Time PCR Assay for 35S Promoter and NOS Terminator

The protocol was an in-house established Real Time-PCR assay using the primers of Lipp et al. (1999) and Trapmann et al. (2002) Real Time PCR targets the 35S promoter and the NOS terminator genes amplifying a 180bp and a 83bp fragment respectively. Reactions were performed in a 25 μL final volume, containing 12.5 μL of Master Mix (KAPA SYBR GREEN Fast qPCR, KAPA BIOSYSTEMS). 0.9 μM of each primer, and 7.5 μL of eluted DNA to make up for 25 μL. Amplification conditions consisted of a 10 min initial denaturation step at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 60 s annealing and elongation at 60°C.

2.3.3 Standard Curves for Real-Time PCR Analysis

The Step One plus^{TM} RT-PCR System (Applied Biosystems) was used for the PCR assay. RT fluorescence measurements were compiled in every cycle. All reactions included negative controls containing the amplification master mix and dH_2O that was used for reagent preparation.

For positive controls and DNA quantification, a standard curve was designed using known concentrations (KC) of DNA extracted from 2% transgenic maize or soybean (KC_1=70 ng/100 mg of food, KC_2=7.0 ng/100 mg of food, KC_3=0.7 ng/100 mg of food, KC_4=70 pg/100 mg of food, KC_5=7.0 pg/100 mg of food, KC_6=700 fg/100 mg of food. A cycle threshold value (Ct) was defined as the cycle of the RT-PCR at which a significant fluorescence increases in comparison to the negative control and the blanks were detected; this increase is associated with an exponential growth of PCR product during the log-linear phase. RT-PCR runs were acceptable only when the negative control had an undetectable Ct, the KC_2 and KC_3 had Cts between 25 and 27, and the efficiency of the PCR was 90-100%. The intrinsic detection limit of the improved method was 0.007 ng (Ct =32). The samples were characterized as negative with a higher Ct. All samples were tested neat and diluted 10^{-1} in dH_2O for the detection of inhibition. Inhibition was defined as a positive PCR result with a diluted specimen, while a negative PCR result was obtained with the specimen tested undiluted.

2.3.4 Repeatability and Reproducibility of Assays

In order to evaluate the repeatability and reproducibility of the method, 5 samples at a concentration of 1.5 ng/μL were randomly chosen as PCR templates and amplified in triplicate in an experiment performed 3 times.

3. Results and Discussion

3.1 Real Time PCR Assay

The specificity of the primer pair was confirmed by Real Time PCR amplification of the peanuts, sesame and hazelnut kernels which were used as negative controls. No amplification signal was observed for any of them even at threshold cycle (CT) values higher than 35. All five levels positive controls (serial dilutions) produced fluorescence curves except the blank control. Real Time-PCR runs were acceptable only when the negative control had an undetectable Ct, and the positive controls had Cts between 25 and 27, and the efficiency of the PCR was 90-100%.

3.2 Repeatability and Reproducibility of Assays

The results of the assay showed that the coefficient of variation values for both intra-experimental and inter-experimental data ranged from 0.45 to 0.80% and 0.23 to 0.71%, respectively (Table 1). These results indicate that Ct measurements were highly reproducible and repeatable, demonstrating the applicability of Real-Time PCR method for the qualitative detection of GMOs.

Table 1. Coefficient of variation values for intra- and inter-experimental test results of 5 randomly chosen samples

	Experiment 1		*Experiment 2*		*Experiment 3*		*Intra-experimental*	
Sample	Mean Ct	CV %	Mean Ct	CV%	Mean Ct	CV%	Mean Ct	CV%
1	25.72 ±0.15	0.58	25.45 ±0.18	0.71	25.68 ±0.10	0.39	26.89 ±0.12	0.45
2	24.74 ±0.10	0.40	24.00 ±0.08	0.33	26.10 ±0.12	0.46	24.98 ±0.20	0.80
3	25.99 ±0.08	0.31	24.45 ±0.15	0.61	25.38 ±0.18	0.71	26.58 ±0.16	0.60
4	26.57 ±0.06	0.23	26.95 ±0.20	0.74	25.52 ±0.15	0.59	25.99 ±0.18	0.69
5	26.01 ±0.14	0.54	26.45 ±0.18	0.68	26.58 ±0.20	0.75	25.75 ±0.15	0.58

3.3 Food Testing

Qualitative PCR-based methods were used to evaluate genetically modified maize and soya products sold commercially in the Attica region (Greece). The majority of the tested samples were maize products, as indicated in Tables 1-3. Both maize and soya foods were mainly cereal based foods, followed by snacks and biscuits.

By using the Real Time PCR method for the identification of lectin gene of soya, all 30 products containing soya were positive and all 40 corn samples were negative. Furthermore, all 40 corn products declared to contain corn were found positive amplifying the maize gene. The analytical sensitivity and specificity was 100% for both genes. In the first category of the corn-based products (n=40), 10 samples (25%) (8 from cereal based products and 2 from snacks) were positive when amplified by the 35S promoter primer, which is specific to the 35S promoter originating from CaMV virus used in genetically modified plants and 3 samples (7.5%) from cereal

based products were detected with both the 35S and the NOS Terminator primer. In total, 13 (32.5%) events of genetic modification (either 35S or NOS terminator) were detected in the corn-based samples (Table 2).

In the second category of the soya-based products (*n*=30), 8 samples (26.7%) all from cereal based products were amplified by the 35S promoter primer and 5 samples (16.7%) were amplified both by the 35S and the NOS Terminator primer. In total, 13 (43.3%) events of genetic modification (either 35S or NOS terminator) were detected in soya samples (Table 2). Overall, in all analyzed samples (*n*=70), a total of 26 events of genetic modification (37.1%) were detected by RT-PCR corresponding to 18 products (25.7%). From the 26 genetically modified events detected 24 (92.3%) were from cereal based products and 2 (7.6%) from the snacks category.\The 35S promoter, a sequence from a cauliflower virus, and the NOS terminator, a sequence from a plant bacterium (*Agrobacterium tumefaciens*), are commonly used as regulation sites, i.e., the "on" and "off" switches for the newly inserted genes. They are present in virtually all genetically modified plants, so most screening methods search for these sequences. Semi-quantitative tests will detect GMOs and compare them to external reference standards of known GMO concentration, with a detection limit of 0.1%. Real-time polymerase chain reaction (PCR) is used for greater accuracy in quantitation and has a detection limit of 0.01%. (Hardegger, Brodmann & Herrmann, 1999)

Table 2. Food product types and GMOs detection indicated by the amplification of 35S promoter and NOS terminator by RT-PCR

Food product category	No. of tested products	declared "*contain corn*"	*amplified of 35S promoter*	*amplified of NOS terminator*	declared "*contain soya*"	*amplified of 35S promoter*	*amplified of NOS terminator*
Cereals	40	25	*8*	*3*	15	*8*	*5*
Biscuits	10	-	-	-	10	-	-
Snacks	20	15	*2*	-	5	-	-
Total	70	40	*10*	*3*	30	*8*	*5*

Table 3. Analytical results of the positive samples for RT-PCR assay of the corn specimens that amplified the 35S promoter or NOS Terminator

No	Specimen	RT-PCR 35S	RT-PCR		
			RT-PCR NOS	DNA yield (ng/100 mg food) Mean Value	Ct Mean Value for positive samples
1	Corn flakes	Positive	Positive	0.784	25.00
2	Corn flakes with fruits	Positive	Negative	0.535	26.10
3	Corn flakes with chocolate	Positive	Negative	0.183	27.32
4	Corn flakes with nuts	Positive	Negative	0.655	26.00
5	Corn flakes 2	Positive	Positive	0.432	26.80
6	Corn flakes bar1	Positive	Negative	0.122	27.12
7	Corn flakes bar2	Positive	Negative	0.235	26.80
8	Corn flakes 3	Positive	Positive	0.635	26.00
9	Snack 1	Positive	Negative	0.056	28.58
10	Snack 2	Positive	Negative	0.068	28.00

Table 4. Analytical results of the positive samples for RT-PCR assay of the soya specimens that amplified the 35S or the NOS promoter

No	Specimen	RT-PCR 35S	RT-PCR		
			RT-PCR NOS	DNA yield (ng/100 mg food) Mean Value	Ct Mean Value for positive samples
1	Cereal flakes with soya 1	Positive	Positive	1.00	25.00
2	Cereal flakes with soya 2	Positive	Positive	0.910	25.30
3	Cereal flakes with soya 3	Positive	Positive	0.900	25.60
4	Cereal flakes with soya and nuts 1	Positive	Positive	0.956	26.00
5	Cereal flakes with soya and nuts 2	Positive	Negative	0.832	26.80
6	Cereal flakes with soya and chocolate 1	Positive	Negative	0.125	27.12
7	Cereal flakes with soya and chocolate 2	Positive	Negative	0.235	26.80
8	Cereal flakes with soya and nuts 3	Positive	Positive	0.635	26.00

The amplicons of the specific sequences detected have been used in the literature to build the different GMOs and regulate expression of transgenes, such as promoter 35S and the terminator NOS (Gachet et al, 1998; Spegelhalter, Lauter & Russell, 2001; van Duijn et al., 2002; Forte et al., 2005; Vijayakumar, Martin, Gowda & Prakash, 2009; Fu et al., 2015). The transgenic content of four commercial food products (maize starch, muesli, lecithin and soybean proteins) has been determined by Vaïtilingom et al. (1999), with the aim to quantify the percentage of "Maximizer" maize and "Roundup Ready" soybean in food products. In the present study, Real Time PCR detected adequately GM maize and soybean food products displayed in the Greek market.

4. Conclusions

The current legislation that regulates the presence of GMOs in crops, foods and ingredients, necessitates the development of reliable and sensitive methods for GMO detection in foods. The objective of the present study was to determine qualitatively by validated PCR methods the occurrence of genetically modified maize and soybean in commercial food products from the Greek market. The result of the study indicated that 26 events of genetic modification (37.1%, *n*=70) were detected by RT-PCR in all analyzed maize and soy products displayed in the Greek market. These products were identified by specific primers and included common GM detection primers for 35S and NOS terminator. The detection of GMOs in more than one third of the investigated food products available in the Greek market indicates the high importance of a qualitative detection system to determine the presence of GMOs in foods. Such real-time screening method could be convenient for the user and applicable to a wide range of food systems. There is a need for a reasoned and rational approach to the benefits of genetically modified foods, as well as consideration of the rights of consumers to know what is in their food. Demand for testing GMOs and for certifying non-GMO foodstuffs has increased dramatically. GMO testing is needed as a precursor to promoting high standards of regulation tracking developments, acting on new evidence and instituting population health.

Acknowledgments

This study was supported by the Fund for Scientific Research FWO-Vlaanderen (grant G.0863.18) and by the KU Leuven Research Fund (Center of Excellence OPTEC-Optimization in Engineering and project C24/18/046).

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