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RESEARCH ARTICLE

Monitoring of Genetically Modified Food and Feed in the Tunisian Market Using Qualitative and Quantitative Real-time PCR

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Abstract Genetically modified organisms (GMO) invade more and more the agricultural production in the world. Although there are no legislations on GM labeling and cultivation of GM crops in Tunisia, the present study aims to check the status of GMO in Tunisian market using qualitative and quantitative real time-PCR (QRT-PCR). About 365 samples were collected and different DNA extraction methods were adapted and optimized. Specific primers targeting 35S promoter from *Cauliflower mosaic virus* (*CaMV*) and nopaline synthase terminator from *Agrobacterium tumefaciens* (*At*) were used for the detection of the GMO insert and Taxon specific primers for the detection of plant species. Validated Taqman® probes (EU-RL) targeting event specific regions of the maize events MON810, Bt11, and the soybean event RRS were used for the quantification studies. Seven food and feed products showed different amounts of RRS (1.9%), MON810 (2.1%), and Bt11 (1.6%). The results demonstrate for the first time the presence of GMO in Tunisian markets reinforcing the need for the development of accurate quantitative methods in routine analyses.

Keywords: biosafety, genetically modified organism, PCR, detection, quantification

Introduction

Genetically modified organisms (GMO) developed for the production of food or feed were placed on the international market since the mid-1990s (1). Products containing or derived from GMO may range from raw commodities to highly processed foods such as cakes and breads. The year 2011, was the 16th year of commercialization of biotech crops, 1996-2011, when growth continued after a remarkable 15 consecutive years of increases; a double-digit increase of 12 million hectares, at a growth rate of 8%, reaching a record 160 million hectares (http://www.isaaa.org/) (2). The growing number of different genetically modified (GM) crops goes in parallel with an increase in the diversity of genetic modifications in commercialized GMOs. This variation in molecular and genetic composition of different transgene events complicates GMO analysis. In order to guarantee consumers' freedom of choice, the use of GMO and GMO derived products in the food chain is subject to precise regulations in several countries. While the labeling of food and derived products is mandatory in the European Union with a threshold of 0.9%, most of the Africans countries are still lacking such regulations. In fact, from the 1 October 2011, food producers, importers, and packagers are required by law, in terms of the Consumer Protection Act and its Regulations, to label GM foods and marketing materials where the GM content is at least 5%. The Table 1 shows the different level of thresholds applied in different continents. North Africa including Tunisia, Algeria, and Morocco has not yet restricted regulations regarding the GMOs with its different uses. Hence, platforms for the development of methodologies for GMO detection and quantification have risen all over the world in order to reveal the adventurous presence of GMO in different matrixes and to comply with respective regulation of labeling.

The detection of GMOs from different matrix may be done by identifying the (or) protein (s) outcome (s) of the transgene or the exogenous DNA itself, that their extraction requires lyses of cells, inactivation of cellular nucleases,

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Table 1. State of art and characteristics of national labeling systems according to the stringency of their regulations

Major country[1)]	Labeling type	Coverage	Threshold level[2)]
European Union	Mandatory	Food, feed, additives, flavorings, and products derived from GM	0.9%
Brazil	Mandatory	Food, feed, meat and animal products, and products derived from GM	1.0%
Venezuela	Mandatory	products derived from GM	1.0%
China	Mandatory	products derived from GM	0.0%
Australia	Mandatory and Voluntary	All products based on content	1.0%
New Zealand	Mandatory and Voluntary	All products based on content	1.0%
Japan	Mandatory and Voluntary	List of food items	5.0%*
Indonesia	Mandatory	List of food items	5.0%*
Russia	Mandatory	All products based on content	0.9%
Saudi Arabia	Mandatory	List of food items	1.0%
South Korea	Mandatory	List of food items	3.0%**
Taiwan	Mandatory	List of food items	5.0%
Thailand	Mandatory	List of food items	5.0%
South Africa	Voluntary	Not specified- All products based on content	5.0%
Phillipines	Voluntary	All products based on content	5.0%
Canada	Voluntary	All products based on content	5.0%
Argentina	Voluntary	Not specified- All products based on content	
United States	Voluntary	All products based on content	N/A
India	Voluntary	primary or processed food, food ingredients, or food additives	
Chile	Voluntary	primary or processed food, food ingredients, or food additives	
Tunisia	N/A (underway)	Not tolerated	
Algeria	N/A	Not tolerated	
Morocco	N/A	Not tolerated	
Egypt	N/A	Not specified	

[1)]United States, for substantial equivalent products only; Brazil, Indonesia, to our knowledge, the labeling regulation has not been fully implemented; Thailand, implemented with "voluntary" enforcement. Penalties are applied in case of reported fraud; Argentina, no specific law; Phillipines, proposed labeling regulation

[2)]*On 3 main ingredients in each product; **On top 5 major ingredients in each product; N/A, not applicable (USDA: http://www.ers.usda.gov, Ref. 15)

and separation of the desired nucleic acid cellular debris (3).

To comply and to anticipate with robust labeling the PCR is widely used as a method of choice in the detection of GMO (4). This is a screening method that allows the detection of regulatory sequences most commonly used in GMO construction, the 35S promoter and the NOS terminator with appropriate primer pairs (5). Transgenic crops invaded more and more countries around the world. Nevertheless, the transgenic foods are severely limited in most regions of the world, especially in Europe, due to the most of people are frightened of consuming transgenic products (6). Most African countries and particularly Tunisia have a significant delay in the field of transgenic plants and have limited access to this technology due to the lack of laws. Genetic modification technology is used in very few African countries, namely South Africa, Zimbabwe, Egypt, Kenya, Burkina Faso, Uganda, and Malawi, and to a lesser extent in Mauritius; of all these countries, only South Africa, Egypt, and Burkina Faso reached the marketing stage (7). The future of genetically engineered foods and crops in Africa will depend heavily on choices African governments make regarding the regulation of this technology (8). Several Arabic countries have conducted survey to monitor the presence of GMO. In Algeria for example, a preliminary screening was conducted on randomly selected 20 samples of crops and 20 food products for the presence of the transgenic 35S CaMV sequence. Results showed that some samples of crops and food products were GMO positive (9). In Egypt study showed that 20% of soy samples contained Roundup Ready™ soybean (RRS); 15% of maize samples were positive for Bt11; and 12.5% positive for Bt11 maize (10) and in Saudi Arabia, a total of 202 samples were tested, 20 products were found to be positive: 16 samples, 3 samples of corn or corn products, and 1 sample of potato (11). In Tunisia biotechnology are expanding, and GMO are gradually becoming a reality for the consumer. However, it is imperative to have methods able to identify and quantify in routine, the content of transgenic DNA in different matrix (raw material, plant leafs, or processed food products), to see if it is below or

above the threshold set in Europe at 0.9% and varies greatly around the world.

Here a new study for the detection and quantification of GMOs in different matrixes collected in the Tunisian market is described. DNA extraction using CTAB method was optimized and adapted for the different samples. The GMO targets used here are P35S (35S promoter issued from *CaMV*), Tnos (Terminator of the nopalin sytnthase gene issued from *At*), and European reference laboratory (EU-RL) validated tests for the quantification of maize events Bt11 and MON810 and soybean event RRS. The taxon specific targets tested are (Adh1) for maize samples, (Lec) for soya samples, (Ugp) for potato samples, (Hord) for barley samples, (Lat) for tomato samples, (Wax) for wheat samples, (Sps) for rice samples, and (Acc) for rapeseed samples. Finally in order to avoid false negatives, Eukaryote specific primers to control the amplificability of the DNA were used.

Materials and Methods

Samples A total of 365 samples were tested. Processed foods Tunisian and imported were gathered from different local markets in some cities in Tunisia, while fresh product was obtained from the Tunisian Cereals Office and the National Agronomic Research Institute in Tunisia (INRAT). These samples contained sweet corn ('Smart Shef', 'Excline', 'Victoria'), corn seed (Egyptian, American, Argentinean, and Tunisian), barley (*Hordeum vulagre*), wheat (*Triticum durum*), rice samples (Uncle Ben's), biscuits samples (Sablé de Retz, Petit Beurre, Poult, Cay, Petit Dej, Sayda, Ti-Salé, and Tuzlu çubuk Kraker), chips (Pringles, Bugles, Chips-Up, Lay's, Crik Crok's Salt, and Crik Crok's Ketchup), cereal samples (Neskuik, Golden Graham's, Grain d'or, and Crok Kid's), tomato samples (Sauce ketchup Bangor, *Solanum lycopersicum*), soya sample (soya sauce "Thai'', oil soybean, and imported feed soybean 'Tourteau'), rapeseed (*Brassica napus*), potato samples (*Solnum tuberosum*), and feed mixture sample. None of these samples were labeled as containing GM ingredients. Depending on the nature of the samples, appropriate storage conditions were provided to store the samples until further use. GM samples (maize event Mon810 and Bt11 and soybean event RRS) were used for relative quantification.

DNA extraction DNA was extracted using the cetyl trimehyl ammonium bromide (CTAB) method with some modifications (12). An amount of 200 mg of grounded samples was transferred into 1.5-mL sterile vials with addition of 1,000 μL CTAB extraction buffer (0.1 M Tris-HCl, 20 g/L CTAB, 1.4 M NaCl, 20 mM EDTA, and pH 8.0) and 20 μL of proteinase K 20 mg/mL was added to the mixture. After Incubation at 65ºC for 120 min, with occasional stirring, a 500 μL of chloroform was added before centrifuging the suspension (10 min, 14,000×*g*, 4ºC). The supernatant were extracted with 200 μL of chloroform and centrifuged (10 min, 14,000×*g*, 4°C). Then, the upper phase was transferred to another tube, mixed with double volume of CTAB precipitation solution (5 g/L CTAB and 0.04 M NaCl) and incubated for 120 min at room temperature. After centrifugation (30 min, 14,000×*g*, 4ºC), the supernatant was discarded and the precipitate was dissolved in 350 μL 1.2 M NaCl, and 350 μL of chloroform, the mixture was mixed for 30 s and then centrifuged (10 min, 14,000×*g*, 4ºC). The upper phase was mixed with the same volume of isopropanol and left at –20ºC for 2 h or overnight. After centrifugation (30 min, 14,000×*g*, 4ºC), the supernatant was discarded and the pellet was dissolved in 500 μL ethanol 70% solution and centrifuged (30 min, 14,000×*g*, 4°C). Finally, the precipitate DNA was redissolved in 30 μL sterile deionized water, and stored at 4ºC.

Screening detection of GMO

***PCR conditions for GMO screening*:** Amplifications by PCR (Gene Amp 7900; Applied Biosystem, Foster City, CA, USA) were carried out in reaction mixtures (25 μL) containing 5 ng/mL of genomic DNA template; 1× polymerase buffer; 2 mM of $MgSO_4$; 200 μM of dNTPs; 1 μM each of forward and reverse primer and 1U/μL of Goldstar Taq according to the following PCR step-cycle program: Pre-denaturation of 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 s, the annealing temperature varies according to the primer for 30 s and extension at 72ºC for 30 s. A final extension at 72ºC for 7 min followed the final cycle for complete synthesis of elongated DNA molecules. The PCR products were analyzed by electrophoresis on a 2% agarose gel. The amplificability of DNA extracted was confirmed using eukaryotic primer TR03 and TR04 l. Identification of the DNA extracted from different samples was carried out by specific reference genes primers listed in while GMO screening test was developed using Tnos primers and P35S primers. All the primer sequences are listed in the Table 2 and 3.

***Amplifiability of sample DNA using plant specific control*:** The presence of soybean and or maize DNA and their amplifiability were confirmed using plant specific primers (13) from soya (lectin gene) and maize (zein gene) after optimization using qualitative PCR and SYBRGreen detection channel in the QRT-PCR (Table 3).

***Agarose gel electrophoresis*:** The extracted DNA was subjected to electrophoresis on a 1% agarose gel at a constant voltage (100 V for 30 min). PCR products were determined on a 2% agarose gel (80 V for 30 min) containing ethidium bromide in 0.5× TBE buffer (Tris, boric acid, and EDTA). The visualization was performed in a UV-transilluminator

and the images were captured with a Polaroid camera and video documentation (Gel Doc; Bio-Rad, Marnes-la-Coquette, France).

***Sequencing of the amplification products*:** The products that were positive after conventional PCR analysis were subject to sequencing, after purification with Wizard PCR Preps DNA purification system (Promega, Fichburg, WI, USA). The same primers used for PCR amplification were labelled with BigDye (ABI PRISM® BigDye TM Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems). The products were separated by gel electrophoresis and analyzed at ABI PRISM 3730XL DNA amplification system (Applied Biosystems). The sequences were aligned with the Genalys® software application software that was used for designed primers.

Quantitative real-time (QRT)-PCR conditions QRT-PCRs were run on an ABI PRISM7900 sequence detection system platform (Applied Biosystems) using the following standard program: 2 min at 50ºC, 10 min at 95ºC, 50 cycles of 15 s at 95ºC, and 1 min at 60ºC. Unless stated, all QRT-PCR were performed in triplicate using the TaqMan® PCR core reagents (Applied Biosystems) in 20 μL final volume. Concentrations of primers and probes were optimized under a range of different MgCl2 concentrations. Each reaction contained: 1× buffer A (including ROX as a passive reference dye); 200 μM each dATP, dCTP, and dGTP; 400 μM dUTP; 1 unit of AmpliTaq gold DNA polymerase; 0.2 units of AmpErase uracil *N*-glycosylase; and 2 μL of DNA solution.

Results and Discussion

Extraction methods and DNA amplification The major step to have reliable PCR results is the use of the adequate extraction method. A good extraction method should generate DNA molecules of sufficient quality and yield. The concentration, purity, and integrity (degree of degradation) of the DNA determine together the suitability for the later analysis. DNA extraction can form a bottleneck in DNA-based GMO analysis, mainly due to the eventual occurrence of inhibition and/or degradation. Inhibition of the PCR reaction due to the presence of contaminants in the analytical sample is a well known phenomenon typical for food matrix, but also resulting from certain extraction buffers or reagents. Inhibitors may be polysaccharides, proteins, lipids, fats, polyphenols, and caramelized sugar or chemicals such as CTAB. A number of common food and feed processing steps like mixing, grinding, extraction, heating, or refining can lead to more or less severe degradation of the analytes rendering them undetectable. This matrix variation requires strategies to be developed on a case-by-case basis, a certain type of method being suitable for a certain type of matrix only. However, raw agricultural products lend themselves best to either DNA or protein analysis. In this study, the CTAB extraction protocol applied to fresh food products has enabled us to have a good quality of DNA. While the extraction of DNA from processed food according to the same protocol is more difficult; but finally, after optimization of the protocols an amplified DNA was obtained.

Once the DNA extracted, the amplifiability of the DNA extracted from the samples was verified using eukaryotic primer TR03 and TR04 through visualization of an amplicons of 137 bp. A band of 137 bp is shown with all samples gathered from various markets in Tunisia. Eukaryotic primer pair targeting the highly conserved sequence 18S-rDNA confirmed that all extracted DNA could be amplified. The DNA extracted from samples was identified using plant specific references genes primers for: maize (Adh1), soya (Lec), potato (UGPase), barley (Hord), tomato (Lat), wheat (Wax), rice (Sps), and rapeseed (Acc) through visualization of the amplicons of 136, 118, 88, 72, 92, 102, 81, and 99 bp, respectively. All the amplification products were validated with sequencing in order to avoid false positives. The presence of potato in the samples was verified by using the (UGPase) primer pair. Results have shown presumed amplicon of 88 bp with 3 potato and 6 types of chips samples (Fig. 1). PCR product is detectable as well in conventional samples and transgenic samples. Existence of barley in samples was verified using (Hord) primer pair. For 9 variety of commercially available barley investigated here, an amplicon of the expected size 72 bp have been obtained in PCR using Hord1/Hord2 primer pair (Fig. 1). To indentify rapeseed samples, (Acc) primer pair was used. An amplicon of 99 bp was obtained in 5 rapeseed available samples using Acc1/Acc2 primer pair. Then tomato samples were analyzed using Lat primer pair. An amplicon of 92 bp has been revealed in 5 tomato samples as well as in the sample of ketch up with Lat 1/Lat2 primer. This primer pair is specific to tomatoes (Fig. 1). The Sps primer pair was used to identify rice samples. The primer pair Sps on the gene for *sucrose-phosphate synthase* used in this study gave a band of 81 bp (Fig. 1). This amplicon demonstrates that this primer pair is specific for rice species identification. For wheat samples, Wax primer pair was used. The revelation of 102 pb band shows that the wheat samples are identified by the primer pair 'Wax' (Fig. 1). The Adh1 primer pair was used to test maize samples. All samples tested were amplified so all contains corn. The primer pair used in this test is specific to maize. The expected band of 136 bp was obtained (Fig. 1). This test confirms the presence of maize in one chips sample as mentioned on the ingredient list. Finally, existence of soya was confirmed with Lec primer pair. This result shows the

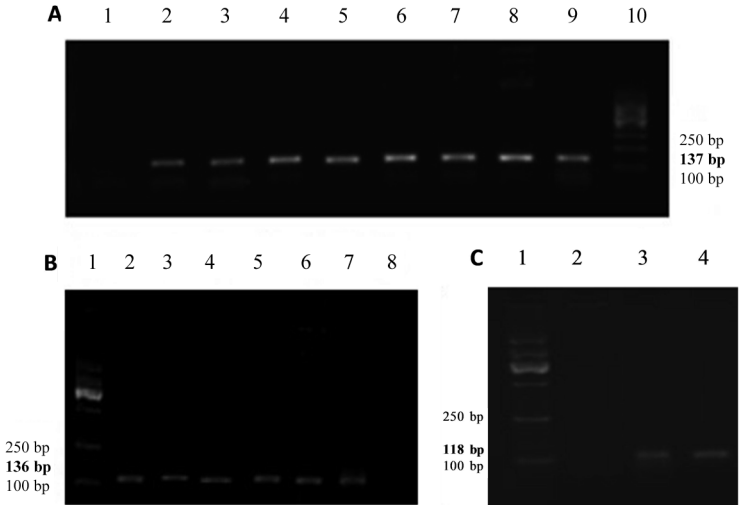

Fig. 1. (A) Lane 1: PCR reagents controls; lane 2 to 9: example of samples tested respectively (sweet corn 'Smart Shef', Egyptian corn seed, biscuit Sablé de Retz, rice Uncle Ben's, chips Pringles, Ketchup Bangor, barley, Neskuik cereal); lane 10: DNA ladder. (B) Lane 1: DNA ladder; lane 2 to 7: examples of maize samples tested respectively (sweet corn 'Excline', sweet corn 'Victoria', sweet corn 'Smart Shef', Chips Bugles, Egyptian variety, American variety); lane 8: PCR reagents control. (C) Lane 1: DNA ladder; lane 2: PCR reagents control; lane 3: Tunisian soybean oil; lane 4: soybean 'Tourteaux'

absence of amplicon with Lec primer pairs, this can be explained as follows that the primer pair used is no specific or that the DNA extracted is very few and has a poor quality which disrupts the efficiency of PCR (Fig. 1). Because of the absence of expected amplicon with some primer pairs under standard conditions were optimized PCR conditions by decreasing the primers concentration, changing $MgSo_4$ concentration, duration of elongation steps, tnumber of cycles, and annealing temperature.

Screening detection of GMO The presence of GMOs in a food matrix was determined by a screening test, targeting 2 regulatory sequences present in most GM construction: Tnos sequence issued from *At*, which marks the polyadenylation site and P35S promoter issued from *CaMV.* Tnos screening test was applied for all samples investigated here. The Tnos screening test was positive for 10 Tunisian imported samples tested (Table 4). The PCR reagents controls demonstrated the absence of contamination

Table 2. List of endogenous references gene primers

Target	Primer	Sequence (5' to 3')	Amplicon size (bp)	Reference	Cycling condition
Maize (*Zea mays*)	Adh1 F Adh1 R	CGTCGTTTCCCATCTCTTCCTCC CCACTCCGAGACCCT CAGTC	136	(16)	35 cycles of 94°C 30 s, 67°C 30 s, 72°C 7 min
Soya (*Glycine max*)	Lec F Lec R	GCCCTCTACTCCACCCCCATCC GCCCATCTGCAAGCCTTTTTGTG	118	(17)	35 cycles of 94°C 30 s, 65°C 30 s, 72°C 7 min
Potato (*Solanum tuberosum*)	Ugp F Ugp R	GGACATGTGAAGAGACGGAGC CCTACCTCTACCCCTCCG	88	(18)	35 cycles of 94°C 30 s, 55°C 30 s, 72°C 7 min
Barley (*Hordeum vulgare*)	Hord F Hord R	AGACAAGGCGTGCAGATCG GACCCTGGACGAGCACACAT	72	(19)	35 cycles of 94°C 30 s, 55°C 30 s, 72°C 7 min
Tomato (*Solanum tuberosum*)	Lat F Lat R	AGACCACGAGAACGATATTTGC TTCTTGCCTTTTCATATCCAGACA	92	(20)	35 cycles of 94°C 30 s, 59°C 30 s, 72°C 7 min
Wheat (*Triticum aestivum*)	Wax F Wax R	GTCGCGGGAACAGAGGTGT GGTGTTCCTCCATTGCGAAA	102	(21)	35 cycles of 94°C 30 s, 55°C 30 s, 72°C 7 min
Rice (*Oryza* sativa)	Sps F Sps R	TTGCGGCTGAACGGATAT CGGTTGATCTTTTCGGGATG	81	(22)	35 cycles of 94°C 30 s, 49°C 30 s, 72°C 7 min
Rapeseed (*Brassica napus*)	Acc F Acc R	TGGTACAATTTGGTTTATATACGGC CTATAACATCAGCCTGTCCAAAAGAAA	99	(23)	35 cycles of 94°C 30 s, 63°C 30 s, 72°C 7 min

Table 3. List of primers used for GMO screening and identification tests

Target	Primer	Sequence (5' to 3')	Amplicon size (bp)	Reference
Qualitative PCR tests				
P35S	P35S F	CGTCTTCAAAGCAAGTGGATTG	79	(24)
	P35S R	TCTTGCGAAGGATAGTGGGATT		
Tnos	Tnos F	TTCTGTTGAATTACGTTAAGCATG	89	(5)
	Tnos R	TTAAATGTATAATTGCGGGACTCTAA		
Quantitative real-time PCR tests				
Bt11	Bt11-ev-f1	TGTGTGGCCATTTATCATCGA	68	http://gmo-crl.jrc.ec.europa.eu/gmomethods/
	Bt11-ev-p1	CGCTCAGTGGAACGAAAACTC		
	Bt11-ev-R1	FAM-TTCCATGACCAAAATCCCTTAACGTGAGT-TAMRA		
MON810	Mail-F1	TCGAAGGACGAAGGACTCTAACGT	92	http://gmo-crl.jrc.ec.europa.eu/gmomethods/
	Mail-P	FAM-AACATCCTTTGCCATTGCCCAGC-TAMRA		
	Mail-S2	GCCACCTTCCTTTTCCACTATCTT		
RRS	40-3-2 AF	TTCATTCAAAATAAGATCATACATACAGGTT	84	http://gmo-crl.jrc.ec.europa.eu/gmomethods/
	40-3-2 AP	FAM-CCTTTTCCATTTGGG-MGBNFQ		
	40-3-2 AR	GGCATTTGTAGGAGCCACCTT		
Universal PCR test				
Eukaryote	TR03	TCT GCC CTA TCA ACT TTC GAT GGT A	137	(25)
	TR04	AAT TTG CGC GCC TGC TGC CTT CCT T		

Table 4. Food and feed samples tested in the present study and results of GMO analysis

Food and feed sample	PCR amplification results[1)]						
	Number of samples analyzed	Plant universal	P35S	Tnos	Maize event Bt11	Maize event MON810	Soybean event RRS
Maize (*Zea mays*)							
Seeds imported from Egypt	5	+	-	-	-	-	-
Grains imported from USA (feed)	5	+	3/5 (+)	-	-	3/5 (+)	-
Seeds imported from Argentina	5	+	-	-	-	-	-
Doux « Smart Shef »	5	+	-	-	-	-	-
Doux « Excline »	5	+	-	-	-	-	-
Doux « Victoria »	5	+	-	-	-	-	-
Tunisian line	5	+	-	-	-	-	-
Doux « Americana »	5	+	-	-	-	-	-
Doux « Daucy »	5	+	-	-	-	-	-
Doux « Sauvers »	5	+	-	-	-	-	-
Doux « Emporium »	5	+	-	-	-	-	-
Barley (*Hordeum vulgare*)							
Line « Ardhawi »	5	+	-	-	-	-	-
Line « Manel »	5	+	-	-	-	-	-
Line « Rihan »	5	+	-	-	-	-	-
Line « Jerbi »	5	+	-	-	-	-	-
Seeds imported from Egypt	5	+	-	-	-	-	-
Tunisian lines	5	+	-	-	-	-	-

and the (+) GMO control indicated the progress of PCR in the right conditions (Fig. 2). The results showed absence of transgenic construct in all samples analyzed. P35S screening test was developed with P35S primer pair. The screening test performed using the P35S primer pair is positive. The expected band of 79 bp in 29 samples was revealed (Table 4 and Fig. 2). The positive GMO control shows that the PCR was carried out in good conditions. The absence of contamination is ensured by the absence of band in the PCR reagents controls. For the remaining samples this test

Table 4. Continued

Food and feed sample	PCR amplification results[1)]						
	Number of samples analyzed	Plant universal	P35S	Tnos	Maize event Bt11	Maize event MON810	Soybean event RRS
			Wheat (*Triticum astivum*)				
Seeds imported from Italy	5	+	-	-	-	-	-
Seeds imported from Turkey	5	+	-	-	-	-	-
Line « Karim »	5	+	-	-	-	-	-
Line « Tanit »	5	+	-	-	-	-	-
Line « Razzag »	5	+	-	-	-	-	-
Line « Salambo »	5	+	-	-	-	-	-
Line « Khiar »	5	+	-	-	-	-	-
Line « Utique »	5	+	-	-	-	-	-
Tunisian lines	5	+	-	-	-	-	-
			Rice (*Oriza sativa*)				
« Uncle Ben's »	5	+	-	-	-	-	-
				Biscuits			
« Sablé de Retz »	5	+	-	-	-	-	-
« Petit Beurre »	5	+	-	-	-	-	-
« Poult »	5	+	-	-	-	-	-
« Cay »	5	+	-	-	-	-	-
« Petit dej »	5	+	-	-	-	-	-
« Sayda »	5	+	-	-	-	-	-
« Ti Salé »	5	+	-	-	-	-	-
« Tuzlu çubuk kraker »	5	+	-	-	-	-	-
			Chips				
« Pringles »	5	+	-	-	-	-	-
Bugles 3D's fromage	5	+	5/5 (+)	-	-	5/5 (+)	-
Bouggles « Smokin' BBQ Flavor »	5	+	5/5 (+)	-	-	5/5 (+)	-
Tortilla Chips	5	+	5/5 (+)	5/5 (+)	5/5 (+)	-	-
« Up »	5	+	5/5 (+)	5/5 (+)	5/5 (+)	-	-
« Lays »	5	+	-	-	-	-	-
« Crik Crok's Salt »	5	+	-	-	-	-	-
« Crik Crok's Ketch up »	5	+	-	-	-	-	-
Up Paprika	5	+	-	-	-	-	-
Up Sour Cream	5	+	-	-	-	-	-
Up Hot Spicy	5	+	-	-	-	-	-
Cheese Fingers	5	+	-	-	-	-	-
Star Sky	5	+	-	-	-	-	-
Up Premium Aged Cheese	5	+	-	-	-	-	-
Sharky	5	+	-	-	-	-	-
Buggles smoked	5	+	-	-	-	-	-
Croki Do's	5	+	-	-	-	-	-
Joy's	5	+	-	-	-	-	-
Sun Chips Paprika	5	+	-	-	-	-	-
Sun Chips Onion	5	+	-	-	-	-	-
Sun Chips Cheese	5	+	-	-	-	-	-
Croki Do's Paprika	5	+	-	-	-	-	-
Golden Chips Salt	5	+	-	-	-	-	-
Sun Frites	5	+	-	-	-	-	-
Golden Chips Paprika	5	+	-	-	-	-	-

Table 4. Continued

Food and feed sample	PCR amplification results[1)]						
	Number of samples analyzed	Plant universal	P35S	Tnos	Maize event Bt11	Maize event MON810	Soybean event RRS
Cereals							
« Nesquik »	5	+	-	-	-	-	-
« Golden Graham's »	5	+	-	-	-	-	-
« Grain d'or »	5	+	-	-	-	-	-
« Crok Kid's »	5	+	-	-	-	-	-
Tomatoes (*Solanum lycopersicum*)							
Ket chup	5	+	-	-	-	-	-
Tunisian line	5	+	-	-	-	-	-
Soybean (*Glycine max*)							
Sauce "Thai"	5	+	-	-	-	-	-
« Daucy »	5	+	-	-	-	-	-
Tourteaux (imported animal feed)	5	+	3/5 (+)	-	-	-	3/5 (+)
Tunisian oil soybean	5	+	3/5 (+)	-	-	-	3/5 (+)
Canola (*Brassica napus*)							
« Line Drakkar »	5	+	-	-	-	-	-
Potatoes (*Solanum tuberosum*)							
Tunisian lines	5	+	-	-	-	-	-
Animal Feed							
Bird feed mixture	5	+	-	-	-	-	-
Total	365	365 (+)	2 (+)	10 (+)	10 (+)	13 (+)	6 (+)

[1)]+, positive amplification; -, negative amplification

is negative. This test indicates the presence of GMO without identifying its nature. These results clearly show that samples marketed in Tunisian markets contain transgenic DNA. For a total of 365 samples tested, 29 were positive for P35S, and 10 for Tnos revealing the presence of GM derived products (Table 4).

Quantification of GMOs in the Tunisian food markets with QRT-PCR All the positive samples of the previous screening tests were tested with primers and probes targeting the edge regions of the maize events MON810 and Bt11 and the soybean event RRS using EU-RL validated methods. All the percentages were calculated using the standard curve method with genomic DNA serial dilutions of the positive controls (100% GMO) maize event MON810, Bt11, and the soybean event RR ranging from 5 to 0.1% (Fig. 3). PCR products are measured at each cycle by means of a target-specific oligonucleotide probe labeled with FAM dye and TAMRA as quencher dye. Standards curves are generated for GMO specific and reference systems by plotting the Ct values measured for the calibration points against the logarithm of the DNA copy numbers and by fitting a regression line into these data; Thereafter the standard curves are used to estimate the copy numbers in the unknown sample DNA by interpolation from the standard curves. For relative quantification of GMO events (Bt11, MON810, and RRS) DNA in a food product sample, the GMO events copy number are divided by the copy number of the reference systems (*adh* for maize and *lec* for soybean) and multiplied by 100 to obtain the percentage value (Fig. 3). The calibration samples were prepared by mixing the appropriate amount of specific GMO event DNA in non GMO DNA to obtain the required percentage with a total of 100 ng of total quantity. As recommended in the European Reference Laboratory (EU-RL), the unit of GMO expression used was haploid genome copy number (HGC). The conversion calculation from ng/μL to HGC was performed using the Arumuganathan and Earle (14) method. The values of the slopes of the standard curves and the R^2 (expressing the linearity of the regression) are summarized in the Table 5. The mean PCR efficiency was 98% for the lectin and adh reference systems and 99% for the specific systems (Bt11, MON810, and RRS). The Table 6 describes an example of the mean Ct values obtained with the calibration curve used for the quantification of the event RR soybean. These data confirms the appropriate performance characteristics of the method tested in terms of efficiency and linearity according to the key documents of the EU-RL (http://gmo-crl.jrc.ec.europa.eu). The 29 out of 365 samples were positive and identified using specific

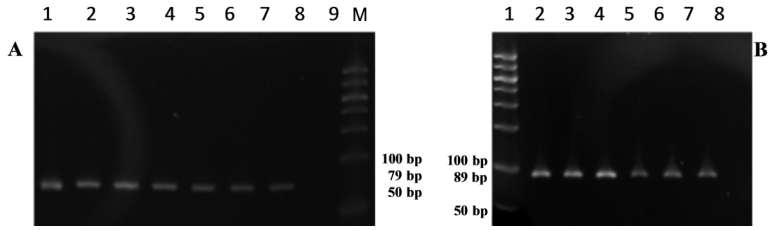

Fig. 2. (A) Lane M: DNA ladder; lane 1 to 7: examples of positive samples tested with P35S primers, respectively [Tourteaux (imported animal feed), Tunisian oil soybean, Bugles 3D's fromage, Buggles <<Smokin' BBQ Flavor>>, Tortilla Chips, Chips « Up » and grains imported from USA (feed)]; lane 8: PCR reagents control; lane 9: DNA ladder (50 bp). (B) Lane 1: DNA ladder (50bp); lane 2 to 7: examples of samples tested respectively with Tnos primers, Tnos/GMO positive control, Chips Up, Tortilla Chips, Tourteaux (imported animal feed), 2 Tunisian oil soybean samples); lane 8: PCR reagents control

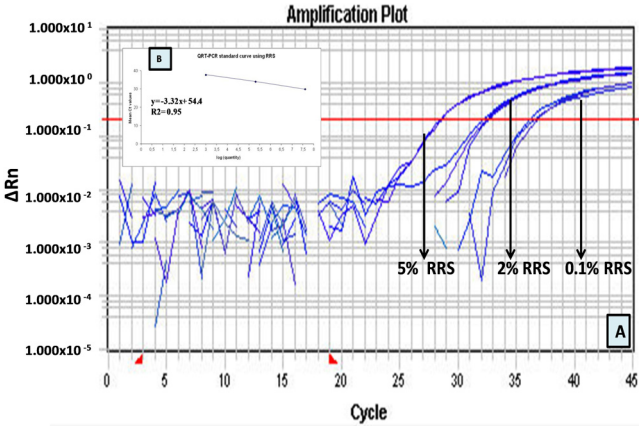

Fig. 3. Example of amplification plot and standard curve for RRS real time PCR assay. A, Amplification plots generated by serial dilution of RRS genomic DNA ranging from 5 to 0.1%; B, calibration curve generated from the amplification data given in A. No amplification observed (Ct=45) with the concentration 0.01% confirming that, the absolute limit of detection of this test is less than 0.1% CEG.

primers as follows: 6 contained the event soybean RRS with an amount 1.9%, 13 the event maize MON810 with an amount of 2.1% and 10 contained the event maize Bt11 with an amount 1.6%. These results showed the urgent need of Tunisian GMO labeling regulations with a fixed mandatory threshold to be implemented for the imported food and feed products in order to offer the free choice for the Tunisian consumers.

Food safety and process control is one of the major subjects to be considered in the near future particularly in the field of GMO analysis. Since no labeling thresholds are implemented in Tunisia and the absence of a strict GMO regulations and directives, GMO screening and quantification are urgently needed for application in routine laboratories. GMOs or derived products may be introduced in our country due to contamination technically unavoidable, due to the import of raw materials and the opening of Tunisia in international markets. Hence, public awareness and legislation in several countries require public notification where food products contain a genetically modified food product. This study is the first in Tunisia to attempt to trace GMOs with an arsenal of molecular biology techniques in different matrix (food, leaves, seeds, etc). It was developed since the absence of strict Tunisian regulations on labeling and traceability of GMOs in products intended for feed and food in order to offer in the near future free choice for consumers between transgenic and conventional products. The results of the screening and QRT-PCR tests developed

Table 5. Values of standard curve slope, PCR efficiency, and linearity (R^2) for the RRS quantification method

Slope[1)]	PCR efficiency (E)	Linearity (R^2)	Slope	PCR efficiency (E)	Linearity (R^2)
Maize event Bt11 (5'junction)			Maize reference system (*adh*)		
-3.34	99%	1.00	-3.37	98%	1.00
-3.40	97%	1.00	-3.34	99%	1.00
Maize event MON810 (5'junction)			Maize reference system (*adh*)		
-3.39	97%	1.00	-3.33	100%	0.99
-3.38	98%	1.00	-3.37	97%	1.00
Soybean event RR (RRS 5' junction)			Soybean reference system (*lec*)		
-3.31	100%	0.99	-3.34	99%	1.00
-3.43	96%	1.00	- 3.32	100%	1.00

[1)]Values of the slopes of the standard curves from which the PCR efficiency is calculated using the formula $[10^{(-1/\text{slope})}-1]\times100$. The mean PCR efficiencies of the GM PCR systems was equal or close to 100%. Linearity of all methods (value R^2) was above 0.99. Overall, data confirm the appropriate performance characteristics of the tested methods in terms of linearity and efficiency.

Table 6. Amplification data of the calibration curve used for quantification of the event RR soybean

Initial template[1)] copies	Signal rate (#positive signals)	Mean Ct-value	SD of observed Ct-value
5%	3/3	28,91	0.085
2%	3/3	32,37	0.105
0.1%	3/3	37,43	0.285
0.01%	0/3	45	-

[1)]Analyses were done on a series of dilutions of 5 to 0.01% of RRS DNA in 100 ng of non GM soybean DNA. Three replicates/dilution were performed and the value of the standard deviation (SD) of the mean Ct-values were calculated for reliable quantification.

showed the presence of GMO in the Tunisian market. These products marketed in Tunisia, are not labeled as containing GMOs or genetically modified ingredients. That's for its essential to establish a regulatory framework regarding the importation and production of GMOs. This suggests that food manufacturers have all the necessary information on primary and secondary material used. It is also essential to test the stability of all transgenic constructs to be placed on the market and have control laboratories to secure use of GMOs.

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