

Development of a Seven-Target Multiplex PCR for the Simultaneous Detection of Transgenic Soybean and Maize in Feeds and Foods

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The detection of genetically modified organisms (GMOs) in food and feed is an important issue for all the subjects involved in raw material control, food industry, and distribution. Because the number of GMOs authorized in the EU increased during the past few years, there is a need for methods that allow a rapid screening of products. In this paper, we propose a method for the simultaneous detection of four transgenic maize (MON810, Bt11, Bt 176, and GA21) and one transgenic soybean (Roundup Ready), which allows routine control analyses to be sped up. DNA was extracted either from maize and soybean seeds and leaves or reference materials, and the recombinant DNA target sequences were detected with 7 primer pairs, accurately designed to be highly specific for each investigated transgene. Cross and negative controls were performed to ensure the specificity of each primer pair. The method was validated on an interlaboratory ring test and good analytical parameters were obtained (LOD = 0.25%, Repeatability, (*r*) = 1; Reproducibility, (*R*) = 0.9). The method was then applied to a model biscuit made of transgenic materials baked for the purpose and to real samples such as feed and foodstuffs. On account of the high recognition specificity and the good detection limits, this multiplex PCR represents a fast and reliable screening method directly applicable in all the laboratories involved in raw material and food control.

KEYWORDS: Polymerase chain reaction; multiplex PCR; GMO; transgenic; maize; soybean; Roundup Ready; Bt176; Bt11; Mon810; GA21

INTRODUCTION

Since GMOs entered the food chain, a scientific and public debate concerning their safety and the need for labeling information arose specially in Europe. At the moment, several transgenes are approved worldwide for cultivation and consumption, while the European Union requires a more strict control to evaluate the potential presence of genetically modified organism materials or their products throughout all the food and feed chains. For this reason, the EU has dedicated special attention to customer information by requiring a compulsory labeling for food products containing more than 0.9% of Roundup Ready soybean and Bt176 maize as specific ingredients (Reg. 49/2000/EEC) and for additives and flavorings derived from these GM crops (Reg. 50/2000/EEC).

In the meantime, other GM products became worldwide available on the market, thus it is of great importance both for

raw material producers and for the food industry to be able to detect and identify the presence of transgenic products or an adventitious contamination throughout all the food chain to comply with the growing request of the customers for a more clear information on the ingredients used for food production. In particular, in the case of organic products, most of the European producers require strict controls to assess the absence of transgenic events in their production.

Although several analytical methods have been proposed (1), routinely used GMO detection methods generally involve specific DNA sequence detection by means of PCR techniques (2), able to detect even small amounts of transgenes in raw materials and processed foods (3, 4).

Furthermore, the availability in the literature of the DNA sequences of some of the transgenes of interest such as maize (5) and gene sequencing facilities have widely opened the possibility of developing new specific PCR methods for a broader range of applications. In particular, the availability of new Taq polymerases allowed to develop new PCR protocols such as multiplex PCR (6): the combination of several primer pairs in the same reaction tube, though reducing the detection limits, would result in a higher number of information in a

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shorter time than single end point PCR and with less consumption of reagents.

Multiplex PCR systems for the screening of raw materials have been recently described, allowing for the simultaneous detection of several GM types of maize (7, 8).

Moreover, given the importance of soybean and its derivatives for the food industry and the worldwide diffusion of transgenic soybean (Roundup Ready), which accounted for 33.3 million of cultivated hectares in 2001 (9), several PCR protocols aiming at identifying the presence of this transgene or its products in raw materials or processed foods have been developed (10, 11).

In the present work, we propose a method based on multiplex PCR to simultaneously detect four types of transgenic maize (MON810, Bt11, Bt176, and GA21), one transgenic soybean (Roundup Ready) and two endogenous controls (the zein gene for maize and the lectin gene for soybean), in raw materials, feeds, and foodstuffs. The choice of the primer pairs and the setting conditions are reported and discussed, as well as the applications to reference materials and real samples.

MATERIALS AND METHODS

Extraction of Genomic DNA. *From Reference Material.* The following commercially available certified reference materials were used: GM-free soybean (IRMM-410S-0, Fluka 83063), GM-free maize flour (IRMM-411, Fluka 63195, Buchs, Switzerland), soy flour containing 5% of Roundup Ready soybean (IRMM-410R, Fluka 44386), maize flour containing 5% of Mon810 maize (IRMM-413-5, Fluka 76182), maize flour containing 5% of Bt11 maize (IRMM-412R-5, Fluka 65944), and maize flour containing 5% of Bt176 maize (IRMM-411, Fluka 17111). Because of the lack of commercially available standard for GA21 maize, 100% GA21 seeds obtained by laboratory greenhouse were milled to obtain a flour. DNA extraction from the reference material was performed with the Wizard method according to the procedure described in the Swiss Food Manual (12).

DNA extract from 100% GA21 maize was diluted with GMO-free maize DNA to obtain a solution containing only maize DNA, with 5% GA21.

From Seeds and Leaves. Seeds and/or leaves of Roundup Ready soybean and MON810, Bt11, Bt176, and GA21 maize were obtained from different research laboratories that grew them and certified them for their purity.

Seeds were treated as follows: 50 g were grounded in an electric mill (Moulinex); DNA was extracted from grounded samples by using the Wizard Plus Minipreps System (Promega) following the manufacturer's specifications with minor modifications (i.e., the quantity of the starting material was 2 g rather than 0.1 g).

Leaves were treated as follows: 300 mg were grounded in liquid nitrogen using mortar and pestle. DNA was extracted following the Doyle JJ and Doyle JL method (13).

Evaluation of the Purity and the Concentration of the Extracts. The DNA concentration was measured by UV absorption at 260 nm, while DNA purity was evaluated on the basis of the UV absorption ratio at 260/280 nm. All the samples showed a 260/280 nm ratio ranging from 1.6 to 1.9. The extracted materials were then diluted to a final concentration of 50 ng/ μ L and used as stock solutions for the following PCR analysis.

Oligonucleotide Primers. On the basis of the published DNA sequences (5, 7, 14) and of the data obtained by sequencing the portion of gene of interest not yet available, we designed different primer pairs to univocally amplify specific DNA sequences for the five transgenes analyzed. The primer design was entirely performed using on line available resources (i.e., the Primer3 software v 0.9) (15). All the primer pairs that successfully amplified their targets were then tested using the Oligos software v 9.7.2 (16) to control their suitability to be used in a multiplex system.

Oligonucleotide primers were purchased as purified and desalted specimen from Sigma Genosys, then diluted to a final concentration of 20 μ M with bidistilled water and stored at -20° C until use.

PCR Conditions. All PCR analyses were performed with a PCR Sprint Thermal Cycler (Thermo Hybaid).

Given the amount of different primers, a so-called *primer mix* was prepared to reduce the analysis-to-analysis variability. To homogenize as much as possible the intensities of all the PCR products, according to different amplification efficiencies, the concentration of each primer pair was accurately calibrated. The primers were premixed to minimize the differences among the primer concentrations due to pipetting variability; the primer mix was prepared at a 4 \times concentration, ready to be diluted during the PCR assembly.

All PCR procedures were performed in a final volume of 25 μ L with the following reagent concentrations: genomic DNA 150 ng, PCR buffer 1 \times (Qiagen GmbH), MgCl₂ 3.5mM, dNTPS 0.4 mM each (Euroclone), primer mix 1 \times , HotStartTaq DNA Polymerase 0.15 U/ μ L (Qiagen GmbH).

Thermal cycler conditions were as follows: preincubation at 95 $^{\circ}$ C for 10 min; 40 cycles consisting of dsDNA denaturation at 95 $^{\circ}$ C for 50 s, primer annealing at 60 $^{\circ}$ C for 50 s, primer extension at 72 $^{\circ}$ C for 50 s; and final elongation at 72 $^{\circ}$ C for 5 min.

Agarose Gel Electrophoresis. PCR products were analyzed using agarose gel electrophoresis. The gel was prepared with 2.0 and 3.0% of agarose (MetaPhor, BioWhittaker 5018, Vallengbaek Strand, DK), for single and multiplex PCR, respectively, in Tris Borate EDTA (TBE) 0.5 \times (Euroclone) with 0.5 μ g/mL of Ethidium Bromide (EtBr) (Euroclone).

The running conditions were constant voltage at 120 V for 1 h in TBE 0.5 \times buffer.

RESULTS AND DISCUSSION

To devise a fast and reliable method for the screening of GM materials, we developed a qualitative PCR system aiming at simultaneously targeting the presence of wild-type maize and soybean, four types of transgenic maize (MON810, Bt11, Bt 176 and GA21), and one transgenic soybean (Roundup Ready). As shown in a parallel work (17), amplicon length seems to be a crucial factor for the detection of DNA in processed foods: DNA degradation during technological treatment can often lead to false negative results due to the presence of highly fragmented DNA. For this reason, the multiplex PCR was set with amplicon lengths no longer than 270 bp to successfully detect the presence of GMOs even in highly processed foods.

Primer Design. Several primers were either designed or taken from the literature and individually tested for their selectivity and efficiency in amplifying their targets.

The design of the primers and the setting of each single PCR were performed using the Primer3 software (15). The primers chosen for the multiplex PCR analysis and their sequence specificity are reported in **Table 1**; their targets and amplicon lengths are reported in **Table 2**.

The possibility of combining all the primer pairs together without generating primer-dimers was evaluated by means of the Oligos software (16). To optimize the multiplex PCR setting (**Table 3**), the primers were chosen on the assumption that only 6 out of 169 possible combinations would generate primer-dimers, and that this eventuality would not really affect the analysis.

PCR Setting. First of all the efficiency of the primers in amplifying the target sequences were separately tested by performing PCRs using each primer pair and the corresponding target genomic DNA (**Figure 1**): as expected, all the primer pairs successfully amplified the DNA sequence of interest, but showed differences in the amplification efficiency, indicating the need for an accurate calibration of the primer concentrations in the multiplex to get comparable amplification responses. A stronger amplification efficiency for the PCRs targeting the two endogenous controls (zein gene and lectin gene) was observed

Table 1. Primers Used for the Multiplex PCR and Their Specificity

primer	sequence (5'-3')	specificity	ref
P-E35S for	CAT TTC ATT TGG AGA GGA CAC G	E35S promoter	
MON810 rev	GCA TTC AGA GAA ACG TGG CAG TA	heat shock protein 70	18
RR rev	TGG GGT TTA TGG AAA TTG GAA	chloroplast transit peptide 4	19
MZ for	CGC CAG AAA TCG TTT TTC AT	zea mays 10-Kda zein gene	
MZ rev	GGT GGT GTC CTT GCT TCC TA	zea mays 10-Kda zein gene	
SL for	ATG GGC TTG CCT TCT TTC T	soybean lectin (Le1) gene	
SL rev	CCG ATG TGT GGA TTT GGT G	soybean lectin (Le1) gene	
Bt11 for	CTG GGA GGC CAA GGT ATC TAA T	intervening sequence 2 (IVS 2)	20
Bt11 rev	GCT GCT GTA GCT GGC CTA ATC T	Phosphinothricin Acetyl Transferase (PAT)	20
Ev176 for	CCC TTC AAC TTC AGC AAC GGC A	Cry1Ab delta-endotoxin	
Ev176 rev	TAG TCG GTC ACG TCG GTC TTC AGG	Cry1Ab delta-endotoxin	
GA21 for	TCT CCT TGA TGG GCT GCA	optimized transit peptide (OTP)	8
GA21 rev	ACG GTG GAA GAG TTC AAT GTA TG	maize 5-enolpyruvylshikimate-3 phosphate synthase (mEPSPS)	8

Table 2. Primer Pairs Used and Amplicon Lengths for Each Target

target	primer pair	amplicon length (bp)
MON 810	P-E35S for MON810 rev	110
Roundup Ready	P-E35S for RR rev	125
maize zein	MZ for MZ rev	139
soy lectin	SL for SL rev	157
Bt 11	Bt11 for Bt11 rev	189
Event 176	Ev176 for Ev176 rev	209
GA 21	GA21 for GA21 rev	270

Table 3. Evaluation of Primer-Dimers Formation^a

primer	primer dimers formation											
1 P-E35S for	+	2	+	+	+	+	+	+	+	+	+	+
2 MON810 rev	1	+	+	+	+	+	+	+	+	+	+	+
3 RR rev	+	+	+	+	5	+	+	+	+	+	+	+
4 MZ for	+	+	+	+	+	+	+	+	+	+	+	+
5 MZ rev	+	+	3	+	+	+	+	+	+	+	+	+
6 SL for	+	+	+	+	+	+	+	+	+	+	+	+
7 SL rev	+	+	+	+	+	+	+	+	+	+	+	+
8 Bt11 for	+	+	+	+	+	+	+	+	+	+	+	+
9 Bt11 rev	+	+	+	+	+	+	+	+	+	+	+	+
10 Ev176 for	+	+	+	+	+	6	+	+	+	+	+	+
11 Ev176 rev	+	+	+	+	+	+	+	+	+	+	+	+
12 GA21 for	+	+	+	+	+	+	+	+	+	+	+	+
13 GA21 rev	+	+	+	+	+	+	+	+	+	+	+	+

^a A "+" indicates the absence of primer-dimers between a specific primer pair; A number indicates the formation of primer-dimers between a specific primer pair.

as well as an unexpected highly efficient amplification of the Bt176 specific PCR: its signal appeared to be comparable to the endogenous control, probably due to the higher T_m of one of the primers.

A mix of DNA extracted from the five transgenes containing 20% of each transgene was then amplified in seven runs (7 PCR processes) using first only one pair of primers starting from the shortest amplicon (MON810 specific PCR, amplicon length 110bp), followed by the addition of a second primer pair and so on, until the last seventh primer pair. The aim was to investigate if there was any eventual primer interference that could result in primer sequestration, thus affecting the final result, and in that case, which primer pair was responsible.

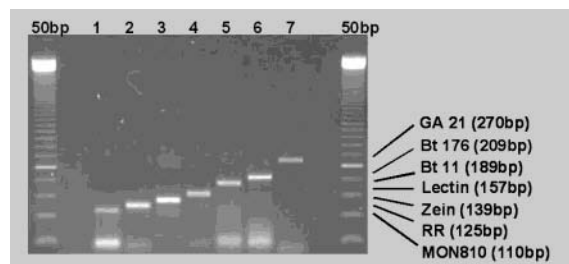


Figure 1. Single PCR amplification of each GMO using its specific primer pair. Lanes 1–7: specific amplification of MON810 maize, Roundup Ready soybean, zein gene, lectin gene, Bt11 maize, Bt176 maize, and GA21 maize.

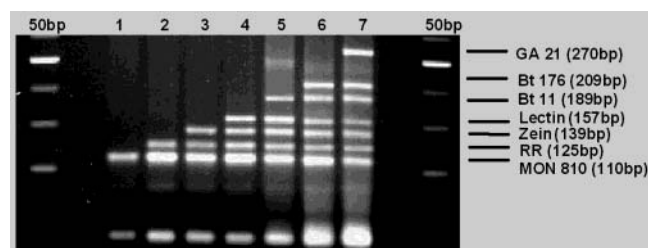


Figure 2. Evaluation of primer-dimers formation. A mix of the DNA extracted from the five transgenes containing 20% of each transgene was amplified using, from lines 1 to 7, one, two, three, four, five, six, and seven primer pairs, following their relative amplicon length.

Furthermore, the experiment was planned to gain information about the presence of eventual unspecific bands due to mispriming. Indeed, no significant reduction of the amplification efficiency appeared for any of the tested PCR and no unspecific amplification was observed in the region of interest, whereas two unspecific signals were present at around 380 and 700 bp (data not shown in figure), not affecting the analyses (**Figure 2**).

To evaluate the correct primer pairs/target response, each transgene was individually tested in a PCR reaction containing all the primer pairs. Thus, a primer mix containing all the primers accurately calibrated was made and used for this assay and for all the following multiplex PCR experiments described in this work. The final concentrations of each primer in the primer mix were 0.2 μ M for MZ for, MZ rev, SL for, SL rev, Ev176 for, and Ev176 rev; 0.4 μ M for MON810 rev, RR rev, Bt11 for, Bt11 rev, GA21 for, GA21 rev; 0.6 μ M for P-E35S for. The mix containing all the primer pairs was then challenged in the PCR reaction with each single transgene; five PCR reactions, one for each transgene, were then performed.

As expected, (**Figure 3**) two bands were simultaneously amplified for each transgene, one corresponding to the endog-

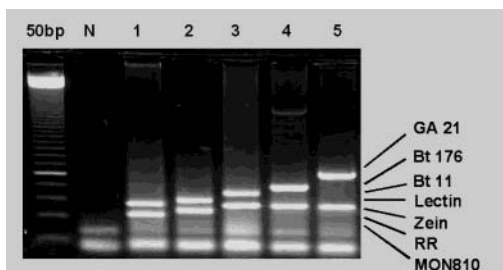


Figure 3. Specificity of the primers designed for the five GMOs. Each transgene was amplified using the multiplex PCR Mastermix. N, negative control (no DNA); line 1–5, multiplex amplification of DNA extracted from MON810 maize (MON810-Zein), Roundup Ready soybean (Roundup-Ready-Lectin), Bt11 maize (zein-Bt11), Bt176 maize (zein-Bt176), and GA21 maize (zein-GA21).

enous control (zein gene or lectin gene) and the other corresponding to the GMO specific amplicon (MON810, RRs, Bt11, Bt176, GA21), confirming the specificity of the primer pairs chosen for each transgene. The homogeneity of all the band intensities also indicated that the primer calibration was correctly performed.

Validation of the Multiplex PCR Method. The multiplex PCR developed underwent a full validation during a ring test trial performed by four independent European laboratories who took part in the test.

The validation parameters that were thought to be important for the validation of such methods were the specificity, limit of detection (LOD), repeatability, reproducibility, and positive and negative deviations. Certified reference materials were used for the evaluation of the detection limits as well as external controls for the analyses of the samples.

Six blind samples having the following composition were prepared: (A) 0.5% Bt176, 0.5% Bt11, 0.5% GA21, 2% Mon810, 2% RRs; (B) 5% soybean, 5% maize, 5% GA21, traces of Bt176; (C) flour containing 1% Bt176; (D) 2% Bt176, 2% Bt11, 2% GA21, 0.5% Mon810, 0.5% RRs; (E) corn muffin mix containing, among others, barley and maize flour, and soya oil; (F) flour containing 0.6% RRs, and maize.

Samples A, B, D, and E were homogenized using an electric mixer. Samples C and F needed no further homogenization. Approximately 1 g of each sample was then distributed in five sterile disposable 15 mL tubes, and the homogeneity of samples A, B, D, and E was checked by taking two 100-mg aliquots from each of the five tubes (10 aliquots per sample were taken). DNA was extracted from the 10 aliquots and quantified by spectrophotometric measurements (12). The DNA extracts were amplified with the multiplex PCR system and the amplification products were analyzed by agarose gel electrophoresis. The results showed that all the samples considered were homogeneous and could then be sent to all the participants for the ring test.

To comply with internationally accepted guidelines for the validation of the analytical methods, all the samples were extracted in duplicate and analyzed in duplicate (4 results for each sample) and one of the samples (A) was extracted five times and analyzed in duplicate to evaluate the repeatability of the method.

For the determination of the LOD, equal quantities of the 5% Bt176, 5% Bt11, 5% GA21, 5% Mon810, and 5% RRs DNA solutions were mixed to prepare a DNA solution containing equal concentrations of each GMO (1%); this solution was then serially diluted (2-fold step) down to a GMO content of 0.08% for each transgene.

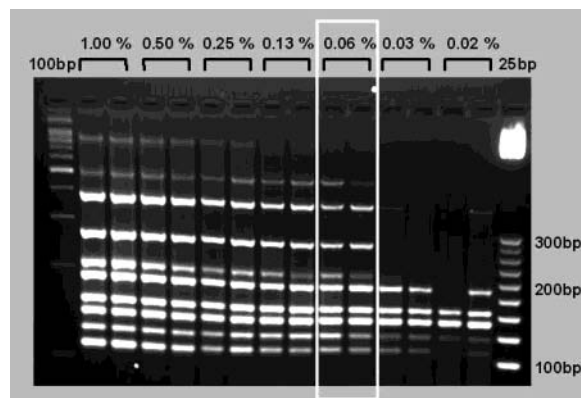


Figure 4. Agarose gel electrophoresis of the PCR amplification products for the determination of the limit of detection (LOD). The different percentages of GMOs were prepared by 2-fold step dilutions starting from a DNA mixture containing equal volumes of 5% DNA extract of each transgene (RR soybean, MON810, Bt11, Bt176, and GA21 maize).

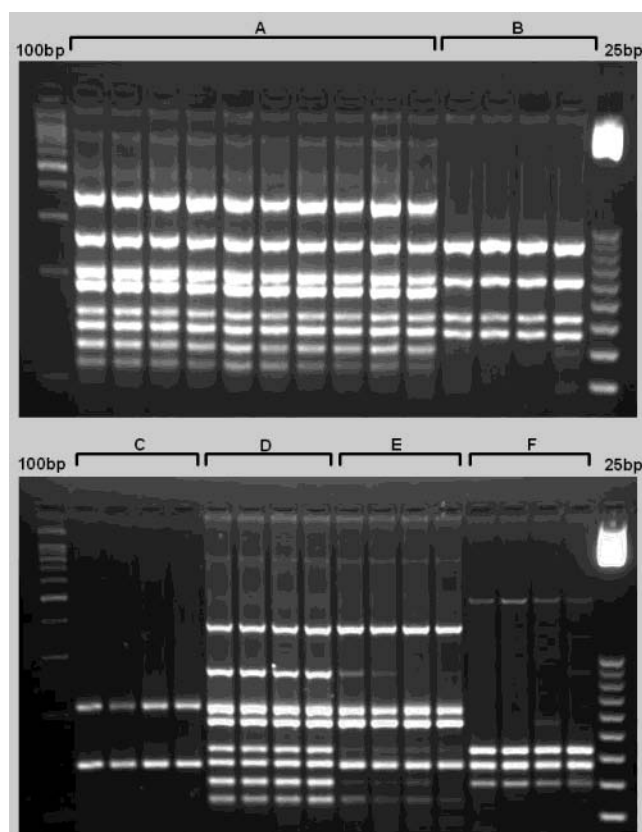


Figure 5. Agarose gel electrophoresis of the PCR amplification products of the blind samples used during the ring test. The composition of each sample was (A) 0.5% Bt176, 0.5% Bt11, 0.5% GA21, 2% MON810, 2% RRs; (B) 5% soybean, 5% maize, 5% GA21, traces of BT176; (C) 1% Bt176; (D) 2% Bt176, 2% Bt11, 2% GA21, 0.5% MON810, 0.5% RRs; (E) commercial corn muffin mix containing soybean oil and traces of transgenic maize; (F) flour containing maize and 0.6% RRs.

The LODs obtained by the different participants ranged from 0.06 (Figure 4) to 0.25%: the latter result, being the highest, was chosen as LOD for the multiplex PCR method.

The analyses of the six blind samples performed by the four groups produced the results used for the determination of the others analytical parameters. In Figure 5, the results of one of the groups are reported.

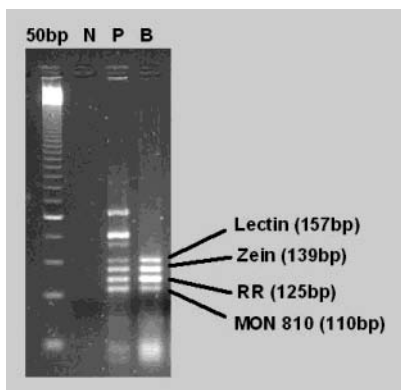


Figure 6. Application of the multiplex PCR to the detection of GMOs in a model biscuit containing known amount of Roundup Ready soybean (15%) and MON810 maize (15%). N, negative control; P, positive control; B, DNA extracted from the biscuit.

Repeatability (r) was measured as the ratio x/n (x = number of correct results under repeatable conditions; n = total number of analyses) and was calculated to be equal to 1.

Reproducibility (R) was measured as the ratio y/n (y = number of correct results under reproducible conditions; n = total number of analyses) and was calculated to be equal to 0.9, which is considered to be acceptable.

Concerning the specificity, the system performed very well, since no amplification signal was obtained for absent traits; however, it has to be mentioned that some false positive results were observed, which were assigned to contaminations.

As for positive deviations, the interpretation of the results showed how hard it is to avoid contamination in such complex systems; special care is to be paid while performing the analyses, to avoid any possible carry over of contamination that could lead to false positive results.

The negative deviation was quite satisfactory: some negative results were produced only by one of the participants, probably generated by a problem with one of the reagents.

In conclusion, all the parameters evaluated were found to be satisfactory enough to consider this multiplex developed for screening purposes as validated; however, more than traditional single PCR, special attention has to be put on avoiding contaminations that could generate false positive results, and external controls have always to be used to distinguish real negative from false negative results.

Application to Foods and Feeds. The method was then tested on real samples to evaluate the eventual matrix effect.

The first food matrix analyzed was a biscuit previously prepared as a model system to study DNA degradation during industrial baking processes (17); this product was made using 15% Roundup Ready soybean flour and 15% MON810 maize flour, then baked according to the usual industrial conditions (180 °C for 20 min). The DNA extracted was tested using the multiplex PCR setting above-reported and the results showed the perfect recognition of all the targets (Figure 6): as expected, the multiplex PCR revealed the presence of the endogenous control both for soybean and maize and the presence of the specific PCR products for RRs and MON810. None of the other transgenes were identified, and no unspecific bands were presents.

Feeds collected directly from production plants were then tested with the multiplex PCR. Maize and soybean samples at different processing stages during feed production were analyzed, together with the final products containing both maize and soybean at different percentages (Figure 7). Samples were

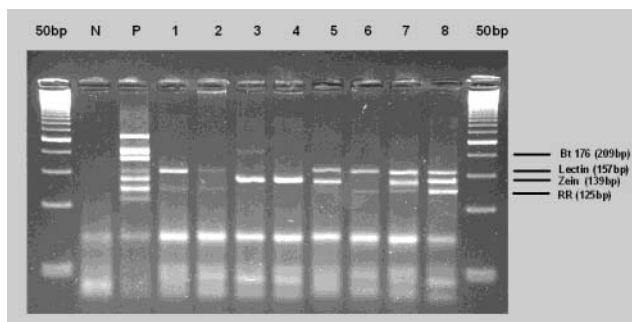


Figure 7. Screening of different feeds to evaluate the presence of possible GMO contaminations. 1, maize; 2, flaked maize; 3, maize pancake; 4, soybean seeds; 5, flaked soybean; 6, soybean pancake; 7, swine feed; 8, fowl feed; 9, bovine feed; 10, bovine TMR; N, negative control; P, positive control.

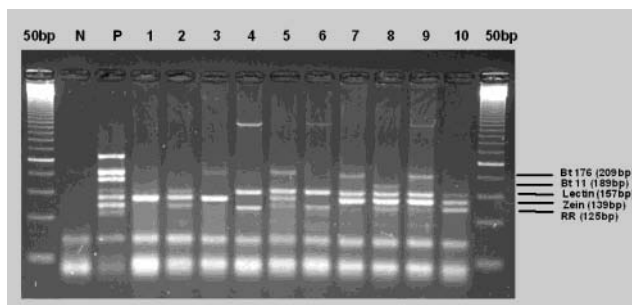


Figure 8. Screening of eight commercial foodstuffs containing various amounts of maize and/or soybean. 1, soybean milk for infants; 2, soybean burger; 3, organic maize biscuits; 4, maize nacho chips; 5, organic soybean biscuits; 6, soybean biscuits; 7, gluten free biscuits containing soybean; 8, gluten free biscuits containing soybean and maize; N, negative control; P, positive control.

chosen from a large number of products analyzed on account of their positive or ambiguous results after a routine CaMV/35S screening. The test revealed the presence of Roundup Ready soybean in seven samples (2, 4, 5, 6, 8, 9, 10), Bt176 maize in four samples (3, 5, 7, 9) and Bt11 in one sample (8). As it can be seen in the figure, in all except one sample, the presence of GMO material was clearly identified.

Different commercially available foodstuffs containing maize and/or soybean were tested, concentrating on those samples that gave positive or ambiguous results with CaMV/35S screening: 8 out of a set of 40 commercial samples were chosen to be analyzed with the multiplex PCR (Figure 8). Soybean and maize were correctly identified as mentioned on the labels; the undeclared presence of maize was detected in sample 5 and 7, probably due to the presence of “vegetable proteins” as ingredient. Transgenic material was detected in several samples: Roundup Ready soybean was found to be present in samples 1, 2, 6, 7, and 8; Bt 176 maize was found in sample 3.

To evaluate the detection limits obtained, the same foodstuffs were tested with a standard real time PCR protocol (21) to determine the content of Roundup Ready soybean; the results showed that all the samples containing Roundup Ready soybean were correctly identified and in particular the quantifications were as follows: 0.50% (± 0.12) for sample 1, 0.60% (± 0.21) for sample 2, and 0.15% (± 0.05) for samples 6, 7, and 8.

The multiplex PCR procedure here proposed appears to be a valid method for the simultaneous detection of soybean, maize, RR soybean, and MON810, Bt176, Bt11, and GA21 maize, in one PCR reaction.

The analytical parameters obtained during the ring test testify a good specificity and sensitivity of the method.

Although the method is purely qualitative, it allows the screening for the presence of GMOs with a detection limit well below the European regulations requirements, as confirmed by the certified samples used and by parallel real time PCR quantifications for Roundup Ready soy.

Furthermore, the successful amplification of processed foods supports the choice of using short amplicons for this kind of analysis.

Because of its robustness, the method proposed can be considered a general, fast, and reliable way of screening GMOs in raw material, feed, and foodstuffs if combined with a proper DNA extraction method suitable for the matrixes considered. As for single PCRs, in this system, special attention has to be paid on avoiding any possible contamination that could affect the validity of the analyses, and external controls need to be used.

Finally, this multiplex PCR method is most suitable to be used in combination with microarray systems, to produce an even faster GMO screening system avoiding the use of gel electrophoresis (22).

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