Romanian Biotechnological Letters *Vol. 15, No.1, Supplement, 2010*

ORIGINAL PAPER

GMO testing for the presence of Roundup Ready soybean

Received for publication, December 18, 2009
Accepted, February 1, 2010

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Abstract

GMO testing is an essential tool for the implementation of some of the European Union policies on the commercialization of genetically modified organisms, as such or processed as food and feed. Soybean transformation event GTS 40-3-2 (Roundup Ready) still represents an important topic in this context. An integrated testing procedure based on the polymerase chain reaction was employed to analyze some commercially available raw and processed matrices for the presence of Roundup Ready soybean. Qualitative analysis by conventional polymerase chain reaction and quantitative measurement by real-time polymerase chain reaction were both designed and used following the internationally accepted requirements for the official control of genetically modified food and feed. The presence of Roundup Ready soybean was detected only in some of the raw samples (seeds). All methods performed acceptably and are recommended to be used for control purposes. Testing of processed and composite food and feed matrices remains difficult to perform.

Key words: GMO testing, Roundup Ready soybean, real-time PCR, DNA extraction.

Introduction

Since their first commercial release, transgenic crops are spreading more rapidly than any agricultural technology in history (RANEY [1]). Despite the potential benefits of this new technology, public and scientific concerns about the environmental, health and social impact of genetically modified crops have been raised (NAP *et al.* [2]). These different views on genetically modified organisms (GMOs), regarding their release into the environment, cultivation, transboundary movement and particularly, their utilization as food or feed, had caused intense controversy which stressed the need for regulative approaches.

In order to ensure transparency and to meet consumers needs, a new set of strict procedures concerning GMOs was laid down in several EU Directives and Regulations (e.g. Directive 2003/18/EC, Regulation 1829/2003, Regulation 1830/2003). The European Commission identified some major concern issues regarding consumers right to make an informed choice, hence the establishment of new policies such as labeling, traceability and post-market monitoring of GMOs and GMO-derived food products (QUERCI *et al.* [3]). In this context, according to EU legislation, competent test laboratories performing specific molecular analyses are designed to provide the scientific basis needed for some of the above-mentioned policies, especially labeling, to become operational. One of the key elements which has to be considered for labeling purposes is the legal threshold introduced by Regulation 1829/2003 [4]. It is stated that labeling is required when the proportion of GMO-derived material is higher than 0.9% of the food ingredients considered individually, providing that this presence is adventitious and technically unavoidable. The laboratories previously referred to are also intended to function as specialized national test/control laboratories within the EU Member States. The need for our country to implement these methods and policies is obvious.

Providing correct, that is reliable and repeatable, analytical results constitutes the essence of official control activities, such as GMO testing. High quality results are produced when the testing laboratory is able to work in a proper and acceptable way, according to internationally-accepted regulations and guidelines (QUERCI *et al.* [5]).

Most commonly used GMO testing procedures are based on the polymerase chain reaction (PCR), because of its technical advantages and also because of the properties of the anlayte (*i.e.* DNA molecules). There are two main phases of this testing procedure: qualitative (yes/no) analysis and quantitative measurement. The first one is done using the conventional PCR, while quantitative measurements are achieved by real-time PCR, technique considered to be the most powerful tool for quantitative analysis of nucleic acids (KUBISTA *et al.* [6]). This type of procedure allows for the expression of results as percentage of GM DNA, in compliance with EU legal provisions (Commission Recommendation 2004/787/EC [7]).

A general testing procedure based on DNA analysis encompasses the following steps: sampling; sample preparation; DNA isolation; DNA characterization; qualitative testing; quantification of GMO-derived material; expression of results.

For more than ten years GTS 40-3-2 (Roundup Ready) has been the only commercially available GMO derived from soybean. Although GM soybean is still not approved for cultivation in the EU, this transformation event continues to be very important due to imports. In Romania, the situation is more complex as Roundup Ready soybean (RRS) was approved for cultivation until January 2007.

Material and methods

The experimental designs we employed are following all recommendations in the field of GMO testing (e.g. working conditions, replication and repetition, use of appropriate controls and certified reference materials). For details see SISEA and PAMFIL [8].

Samples and sample preparation

Several raw materials (seeds) and processed matrices (textured vegetable protein, vegetarian pâté, soy-based cream cheese alternative, soymilk, tofu, biscuits) containing or consisting of soybean were tested. Certified reference materials (CRM) (*i.e.* ERM-BF410 series – 0%, 0.1%, 0.5%, 1%, 2%, and 5% RRS content) produced by the Institute for Reference Materials and Measurements and some maize samples were also included in the experiments, as positive or negative controls.

Prior to DNA isolation, some procedures (e.g. milling, reduction of size) corresponding to sample preparation, were carried out in a dedicated area. The purpose of proper sampling and sample preparation is to ensure that the test portion is an accurate representation of the whole sample being analyzed. Grinding was done with a Grindomix GM 200 knife mill (Retch). Dried samples were added water in order to improve grinding and extraction.

DNA extraction and purification

Samples were processed using the QIAamp DNA Stool Mini kit (Qiagen), method based on silica gel membrane technology. 100 mg were used as test sample and the extraction was carried out according to the manufacturer instructions for isolation of DNA from stool for pathogen detection.

DNA characterization

The concentration of DNA in the extracts was estimated using a NanoDrop Nd-1000 UV/Vis 1µl Spectrophotometer (Labtech International), while the structural integrity of extracted DNA was evaluated by agarose gel electrophoresis (1%, w/v;TAE system) combined with EtBr staining and UV transillumination (for details see the following section).

In order to evaluate the presence and effect of PCR inhibitors, special amplification runs are carried out on the extracted DNA. The DNA extract is first brought to a level corresponding to the highest DNA concentration intended to be used in the subsequent PCR-based analyses, the so called 'undiluted' sample. From this 'initial' concentration, a fourfold dilution series is prepared (from 1:4 to 1:256) (ŽEL *et al.* [9]; FOTI *et al.* [10]). A real-time PCR assay is

Table 1.

Oligonucleotides used in this study

Name	Target	5'- 3' sequence	Annealing temp. (°C)	Amplicon (bp)	Reference
Lectin-F* Lectin-R* Lectin-TMP* (TaqMan porbe)	*Le1*	TCC ACC CCC ATC CAC ATT T GGC ATA GAA GGT GAA GTT GAA GGA FAM-AAC CGG TAG CGT TGC CAG CTT CG-TAMRA	60	81	DEBODE *et al.* [11] BRODMANN *et al.* in CANKAR *et al.* [12] FOTI *et al.* [10] SLMB in SR EN ISO 21570:2006 [13] TAVERNIERS *et al.* [14]
CP3 CP4	*trnL* chloroplast sequence	GGG GAT AGA GGG ACT TGA AC CGA AAT CGG TAG ACG CTA CG	63	> 500	THION *et al.* [15]
GM03 GM04	*Le1*	GCC CTC TAC TCC ACC CCC ATC C GCC CAT CTG CAA GCC TTT TTG TG	63	118	UJHELYI *et al.* [16] CARDARELLI *et al.* [17] QUERCI *et al.* [18] MEYER and JACCAUD** MEYER *et al.*** ROTT *et al.* [19] THION *et al.* [15] GACHET *et al.* [20]
Lektin1 Lektin6	*Le1*	GAC GCT ATT GTG ACC TCC TC GAA AGT GTC AAG CTT AAC AGC GAC G	60	318	ABDULLAH *et al.* [21] TENGEL *et al.* [22]
p*35S*-cf3 p*35S*-cr4	P-E*35S*	CCA CGT CTT CAA AGC AAG TGG TCC TCT CCA AAT GAA ATG AAC TTC C	62	123	CARDARELLI *et al.* [17] QUERCI *et al.* [18] LIPP *et al.***
CaMV1 CaMV2	P-E*35S*	GAA GGT GGC TCC TAC AAA TGC C GTG GGA TTG TGC GTC ATC CC	63	199	THION *et al.* [15] WOLF *et al.* [23]
HA-*nos* 118f HA-*nos* 118r	T-*nos*	GCA TGA CGT TAT TTA TGA GAT GGG GAC ACC GCG CGC GAT AAT TTA TCC	62	118	UJHELYI *et al.* [16] ABDULLAH *et al.* [21] CARDARELLI *et al.* [17] SR EN ISO 21569:2006 [24] QUERCI *et al.* [18] LIPP *et al.***
RR01 RR04	RRS construct	TGG CGC CCA AAG CTT GCA TGG C CCC CAA GTT CCT AAA TCT TCA AGT	60	356	ABDULLAH *et al.* [21] TENGEL *et al.* [22] KÖPPEL *et al.* in STUDER *et al.* [25]
GM05 GM09	RRS construct	CCA CTG ACG TAA GGG ATG ACG CAT GAA GGA CCG GTG GGA GAT	60	447	ANGONESI BROD *et al.* [26] CARDARELLI *et al.* [17] QUERCI *et al.* [18] GACHET *et al.* [20] STUDER *et al.*[25] MAYER and JACCAUD**
GM07 GM08	RRS construct	ATC CCA CTA TCC TTC GCA AGA TGG GGT TTA TGG AAA TTG GAA	60	169	ANGONESI BROD *et al.* [26] CAZZOLA and PETRUCCELLI [27] QUERCI *et al.* [18] GACHET *et al.* [20] STUDER *et al.* [25] MEYER and JACCAUD**

* Used only for inhibition control by real-time PCR.
** Cited in QUERCI and MAZZARA [28].

then conducted with the species specific (reference) system. This means that a taxon specific (reference) gene is used as target for the PCR. For soybean the *lectin* (*Le1*) gene is usually used as reference (ŽEL *et al.* [9]). These real-time PCR experiments were run on a

LightCycler 480 platform (Roche), using a universal TaqMan master mix (*i.e.* LightCycler 480 Probes Master, Roche) and appropriate primers and probe (Table 1). The final reaction mixture contained: 0.9 µM of each primer; 0.1 µM TaqMan probe; 1X Probes Master; ddH_2O up to a final volume of 18 µl; 2µl of DNA solution (up to 50 ng/µl). Amplification parameters were as follows: step 1, incubation (enzyme activation) at 95 °C for 10 min; step 2, denaturation at 95 °C for 15 s, annealing at 60 °C for 25 s, and elongation at 72 °C for 10 s; step 3, conservation at 4 °C. Step 2 is repeated 45 times. For this step, 'Quantification' analysis mode is selected. The fluorescent signal is acquired during each elongation phase in 'Single' acquisition mode. Standard curve was constructed using the 'Fit Points' algorithm.

In order to evaluate the inhibiting effect, the C_T values of the diluted samples are plotted against the logarithm of the dilution factor, and an equation is calculated by linear regression. According to the European Network of GMO Laboratories (see ENGL [29]) and ŽEL *et al.* [9], three criteria have to be met: the slope of the regression line should be within -3.6 and -3.1 and the linearity (R^2 coefficient) should be above 0.98; finally, the difference (ΔC_T) between the C_T value for the 'undiluted' sample, extrapolated from the linear regression, and the measured C_T for the same sample, should be within 0.5.

Amplificability of the isolated DNA solutions could also be checked by conventional PCR, again employing the soy-specific (endogenous) *lectin* gene as target. The procedure is described in the next section.

Qualitative testing

PCR primers (Table 1) were chosen to detect three types of target sequences, each corresponding to one of the qualitative testing stages: taxon-specific DNA detection; GMO screening; identification of transformation events. All primers were used with the same universal amplification protocol. The GoTaq Flexi DNA Polymerase kit and the dNTP Mix, both from Promega, were used to obtain the following PCR master mix for each reaction: 1X GoTaq Green Reaction Buffer; 2.5 mM $MgCl_2$; 0.2 mM dNTPs mix; 0.5 µM of each primer; GoTaq Flexi DNA Polymerase 0.03 U/µl; ddH_2O up to a final volume of 23 µl; 2 µl of DNA solution. The reactions were set up at low, positive temperature (0-4 °C) in a vertical laminar flow cabinet, in order to prevent any unwanted interactions between reagents and to avoid contamination. The amplification program had the following parameters: step 1, initial denaturation at 95 °C for 3 min; step 2, denaturation at 94 °C for 30 s, primer-specific annealing temperature (see Table 1) for 30 s, elongation at 72 °C for 30 s; step 3, final elongation at 72 °C for 3 min; conservation of the PCR amplification products at 4 °C (no time limit). Step 2 is repeated for 40 cycles. The amplifications were run in a Palm Cycler apparatus (Corbett Research).

The results of the PCR reaction were assessed by agarose gel (2 %, w/v; TAE system) electrophoresis combined with EtBr staining and UV transillumination. Biotools MB Agarose was used and the gels were stained in 0.01 mg/ml EtBr solution obtained by diluting the EtBr stock (10 mg/ml) in 1X TAE electrophoresis buffer. Several DNA ladders produced by Promega were used throughout the experiments: Benchtop 100bp DNA Ladder; 100bp DNA Step Ladder; Benchtop PCR Markers.

Quantitative testing

For this analysis we used the Biogenics RoundUp Ready Soya QT (Biotools) in combination with the Rotor-Gene 3000 instrument (Corbett Research)was used. The experimental design followed the principles of the 'standard curve' approach, an absolute concentration being calculated for each of the two target sequences (*i.e.* transgene and endogene, respectively) and the ratio between the two representing the concentration of GM

material in the sample. The working protocol proposed by the manufacturer was slightly modified in terms of master mix composition and standard curve construction. With respect to the master mix, only the volume of DNA polymerase was reduced to 1 µl, the difference being supplemented with ddH_2O. For the construction of the standard curves, five levels of target molecules were set as five-fold serial dilutions of the calibration solution included in the kit (PC SOYA). Accordingly, the reference (endogene) calibration system contained 400.000, 80.000, 16.000, 3.200, and 640 target molecules/µl, while the transgenic one had 20.000, 4.000, 800, 160, and 32 target molecules/µl. All measurements were used to assess method performance, the following parameters being calculated: mean, error (bias) (%), repeatability standard deviation (SD_r), and relative standard deviation of repeatability (RSD_r).

Results

DNA characterization

High concentrations of DNA were obtained from CRMs, textured soybean protein, and seeds, the average values being 265 ng/µl, 245 ng/µl, and 220 ng/µl, respectively. The average values of A_{260}/A_{280} and A_{260}/A_{230} ratios were also very similar for all three groups (data not shown). Nevertheless, significant differences were noted when analyzing the structural integrity of the extracted DNA (Figure 1).

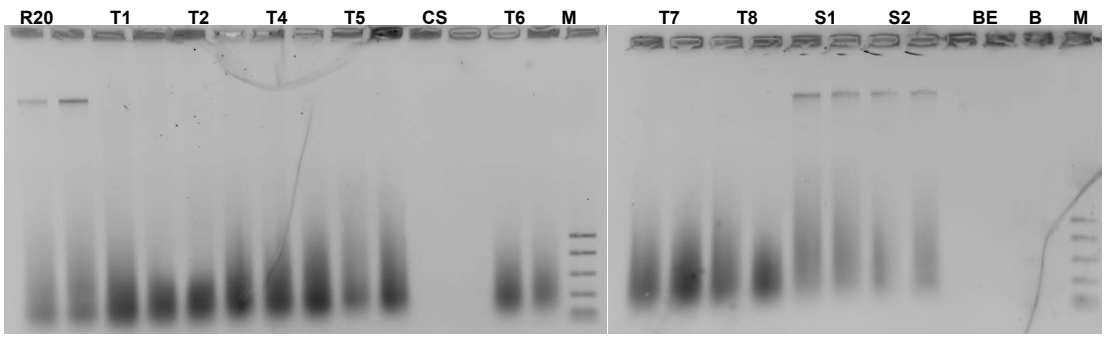


Fig.1. Gel electrophoresis of DNA extracts obtained with the QIAamp DNA Stool Mini Kit from samples containing soybean. Samples: S, soybeans; R, CRM; T, textured soy protein; CS, soybean-based cheese analogue; BE, energy bar; B, extraction blank.

Extracts obtained from processed samples had low concentrations of DNA and the nucleic acid molecules were highly fragmented.

Initially, all extracts were check for inhibition, using the conventional PCR procedure. The results are presented in the following section. Inhibition check by real-time PCR was performed only on the samples identified as GMO positives in the qualitative testing phase. No significant inhibitory effect was observed.

Qualitative testing (PCR analysis)

In order to check DNA amplificability, all extracts were first tested with the taxon specific primers. This analysis confirmed the presence of soybean-derived DNA in each sample, thus eliminating the possibility of getting false negative results in the subsequent analyses due to PCR inhibition.

GMO specific primers held positive results only in the case of some raw soybean samples. The findings were consistent for all screening and RRS specific primers. Also, all control samples produced the expected results confirming the correctness of results.

Some of the qualitative testing results are illustrated in Figures 2 to 5.

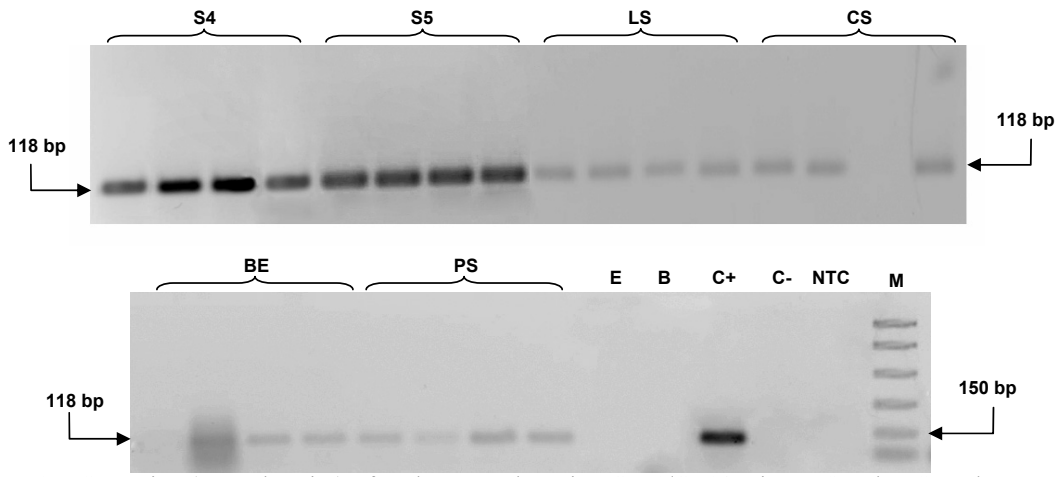


Fig. 2. PCR testing (taxon detection) of soybean samples using GM03/GM04 primers. Samples: S, soybeans; PS, vegetarian pâté containing soybean; CS, soybean-based cream cheese analogue; BE, energy bar; LS, soymilk; E, environmental control; B, extraction blank; C+, positive DNA target control; C-, negative DNA target control; NTC, amplification reagent control; M, DNA ladder.

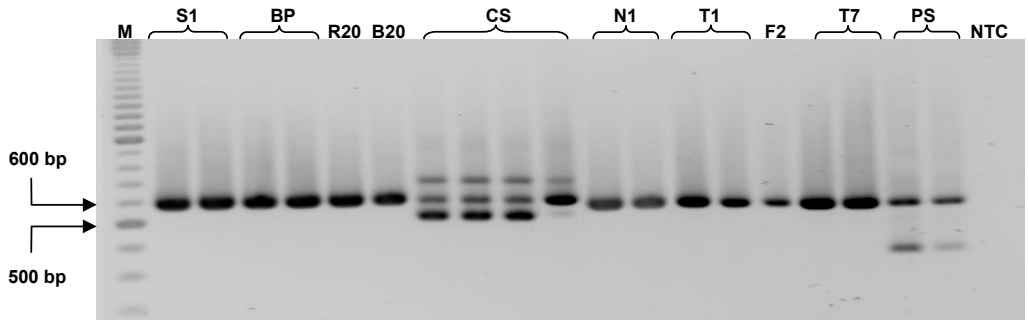


Fig. 3. PCR testing of maize samples using CP3/CP4 primers. Samples: M, DNA ladder; S, soybeans; BP, canned maize kernels; R20, RRS CRM; B20, BT176 maize CRM; CS, soybean-based cheese analogue; N, maize flour; T, textured soy protein; PS, vegetarian pâté containing soybean; F, cornflakes; NTC, amplification reagent control.

Quantitative testing (real-time PCR analysis)

The quantitative analysis was performed only on the unknown samples identified as positives in the previous analytical phase and also on some of the soybean CRMs. Figure 6 is showing the data resulted from a quantification experiment. All reactions met the minimum performance requirements set for this kind of analyses (e.g. $R^2 \geq 0.98$ and $-3.1 \geq \text{slope} \geq -3.6$).

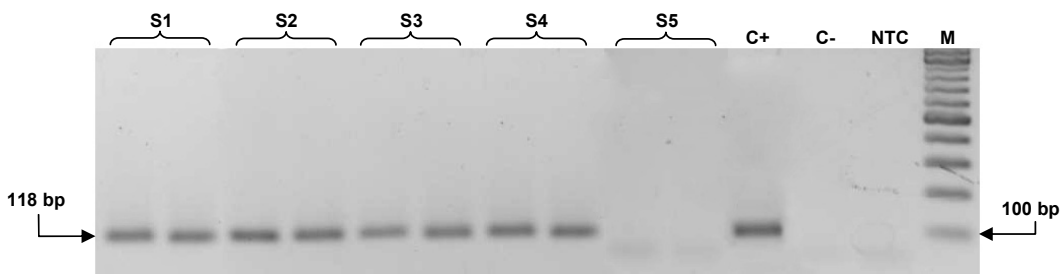


Fig. 4. GMO screening using primers specific for the *nos* terminator (HA-*nos* r/HA-*nos* f). Samples: S, soybeans; C+, positive DNA target control; C-, negative DNA target control; NTC, amplification reagent control; M, DNA ladder.

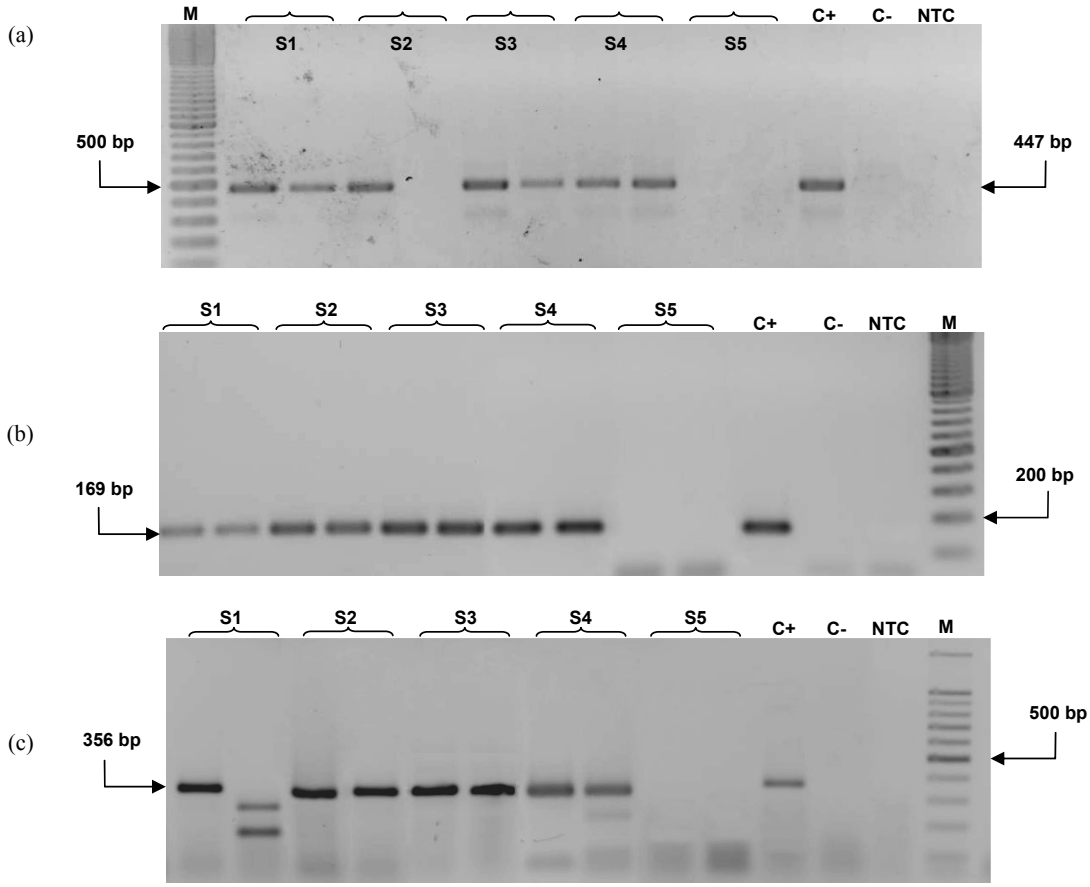
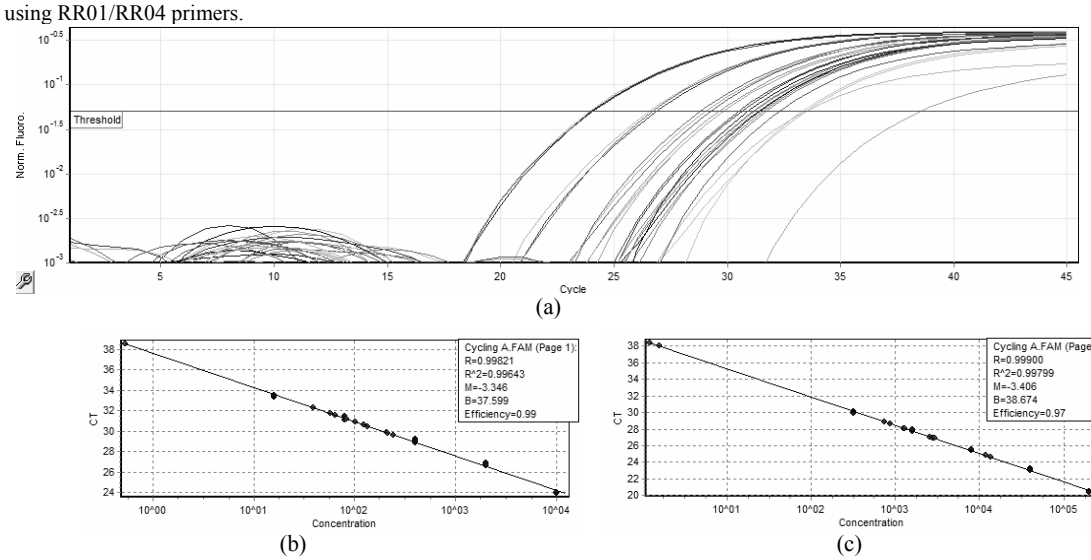
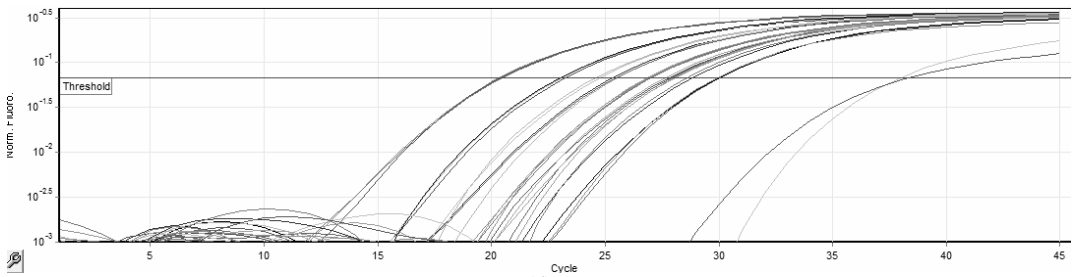


Fig. 5. Specific detection of Roundup Ready soybean. Samples: S, soybeans; C+, positive DNA target control; C-, negative DNA target control; NTC, amplification reagent control; M, DNA ladder. (a) Results of PCR amplification using GM05/GM09 primers. (b) Results of PCR amplification using GM07/GM08 primers. (c) Results of PCR amplification using RR01/RR04 primers.





(d)

Fig. 6. Detection and quantification of event GTS 40-3-2 using the Rotor-Gene 3000 apparatus. (a) Amplification plot for the transgenic system. Calibrators are represented by blue dots, while unknown samples correspond to red dots. (b) Standard curve generated for the transgenic system. (c) Standard curve generated for the reference system. Calibrators are represented by blue dots, while unknown samples correspond to red dots. (d) Amplification plot for the reference system.

The results of the analyses and the calculated statistical parameters are given in Table 2. According to ENGL minimum method performance requirements (ENGL [29]), over the whole dynamic range, bias should be within ± 25% of the accepted reference value and RSD_r should fall below 25%.

Discussion

DNA characterization

The isolation procedure is important for getting accurate and correct final results because it is directly responsible for the characteristics of the DNA extract, especially purity (absence of inhibitors). Thus, assessment of extracted DNA is important in order to evaluate the suitability of the extracted DNA for PCR amplification. Spectrophotometer measurements and/or agarose gel electrophoresis were generally employed for this task, but the most useful is the PCR inhibition check. Traditionally, this was done in the first testing phase (*i.e.* qualitative analysis), corresponding to the so-called 'taxon-detection'. However, a conventional PCR amplification will not identify partial inhibition of the reaction, and because of this, even if the costs are higher, we recommend the employment of a real-time PCR experiment which would permit a more accurate assessment.

Table 2.

Trueness and repeatability of the quantitative method

rt-PCR analysis and data processing		Results			
Sample		R10	R20	R50	S1
Measurement	1	1.02	1.89	5.63	57.93
	2	0.97	2.09	5.04	64.21
	3	1.17	2.21	5.43	63.14
	4	-	-	4.55	-
Theoretical GMO content (%)		1	2	5	-
Mean		1.053	2.063	5.163	61.67
Error (bias) (%)		5.333	1.666	3.25	-
Repeatability standard deviation (SD_r)		0.104	0.162	0.476	3.36
Rel. std. dev. of repeatability (RSD_r)[1]		9.881%	7.834%	9.224%	5.44%

[1] Obtained as ratio between standard deviation and mean.

Qualitative testing

Qualitative GMO testing negative results could be explained in four ways: the absence of GM ingredients; the initial presence of GMO-derived material in low quantities, below the limit of detection (LOD); the initial presence of GMO-derived material in sufficient quantities

for detection, but the analyte (*i.e.* DNA) is degraded during processing, making it useless/unavailable for the analytical process; the employed methods are not fit for purpose (their performances are low). Although the methods we used were not completely *in-house* validated, we are confident that reported results are correct.

As stated before, a general amplification procedure was created and used for all primers. This protocol produced satisfactory results which also constitute a good measure for the robustness of the original PCR methods. Nevertheless, if certain primer pairs are to be selected, we recommend the use of the original protocols in order to increase the performance of the method.

Quantitative testing

Based on the evaluation of trueness and repeatability we conclude that method performance meets the acceptance criteria listed by relevant sources in this field. Calculated parameters are representative for the whole analysis procedure, as they include the variability related to the extraction step. However, we wish to point out that calculations were based on a small amount of data; also dynamic range was not entirely covered.

Concerning the quantification procedure, we believe that the modified calibration curves are better suited for low GM contents.

Table 3 summarizes the overall results of our analyses.

Conclusions

According to the experiments we performed, the employed protocols are producing valid results, at least in the case of raw soybeans. However, to eliminate all doubts about method performance and fitness for purpose, the *in-house* validation should be completed. Also, the results for the other types of matrices should be double-checked (e.g. spikes analysis) for confirmation.

Generally speaking, we believe that qualitative testing does not pose any substantial problems; on the other hand, correct quantification is quite difficult to achieve, especially in the case of processed and composite matrices (Moriuchi *et al.* [30]; Corbisier *et al.* [31]). In this context, the most important elements are represented by the characteristics of the initial matrix to be tested and those of the isolated analyte.

Other challenges for GMO testing are represented by the increasing number of commercially available transformation events, the suitability of the CRMs, the stability of inserted DNA, the stochastic effects affecting some of the analytical steps etc.

Table 3.

General results of GMO testing

No.	Type of matrix	Code	Sample type	Detection		GMO %
				Taxon-specific	GMO-specific	
1	TVP[1], granules	T1	Unknown	+	-	x
2	TPS, granules	T2	Unknown	+	-	x
3	TPS, granules	T3	Unknown	+	-	x
4	TPS, schnitzels	T4	Unknown	+	-	x
5	TPS, chunks	T5	Unknown	+	-	x
6	TPS, chunks	T6	Unknown	+	-	x
7	TPS, schnitzels	T7	Unknown	+	-	x
8	TPS, chunks	T8	Unknown	+	-	x
9	Raw soybean seeds	S1	Unknown	+	+	63.14[5]
10	Raw soybean seeds	S2	Unknown	+	+	63.47
11	Raw soybean seeds	S3	Unknown	+	+	34.12
12	Raw soybean seeds	S4	Unknown	+	+	76.15

13	Raw soybean seeds	S5	Control[3]	+	-	x
14	Raw soybean seeds	S6	Unknown	+	-	x
15	Soymilk	LS	Unknown	+	-	x
16	Cheese[2]	CS	Unknown	+	-	x
17	Energy bar	BE	Unknown	+	-	x
18	Vegetarian pâté	TF	Unknown	+	-	x
19	Tofu	PS	Unknown	+	-	x
20	Biscuits	BS	Unknown	+	-	x
21	ERM-BF410a	R0	Control[3]	+	-	x
22	ERM-BF410b	R1	Control[4]	+	+	0.089
23	ERM-BF410c	R5	Control[4]	+	+	0.6
24	ERM-BF410d	R10	Control[4]	+	+	1.01[5]
25	ERM-BF410e	R20	Control[4]	+	+	2.03[5]
26	ERM-BF410f	R50	Control[4]	+	+	5.163[6]

[1] Textured vegetable protein.
[2] Soy-based cream cheese alternative.
[3] Control sample positive for conventional soybean and negative for GM soybean.
[4] Control sample positive for both conventional and GM soybean.
[5] Mean value of three independent measurements.
[6] Mean value of four independent measurements.
'+' Target sequence identified (positive result).
'-' Target sequence not identified (negative result).
'x' Analysis not performed.

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