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Detection and quantification of MON89788 soybean in foodstuffs by a new real-time PCR assay

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ABSTRACT

Real-time polymerase chain reaction (PCR) is the technique of choice for genetically modified organisms (GMO) detection and quantification because of its versatility, sensitivity, specificity, and high-throughput applications. This work aimed at developing qualitative and real-time PCR assays to trace Roundup Ready 2 (RR2) soybean in foodstuffs. Event-specific primers targeting RR2 soybean were designed to amplify an expected fragment of 121 bp. A real-time PCR assay with hydrolysis fluorescent probes and the double calibration curve method was successfully developed and validated through an interlaboratory study (GIPSA/USDA). The method was applied to soybean-containing foods, confirming qualitative PCR results. The quantitative analysis showed RR2 soybean contents of 0.04-0.13% in 3 food samples and one containing 2.89%, above the mandatory threshold for labelling of foods (0.9%) (Regulation No 1829/2003).

1. INTRODUCTION

Since the beginning of genetically modified organism (GMO) commercialization, the global area of biotech crops has increased 100-fold, from 1.7 million hectares in 1996 to 179.7 million hectares in 2015. Soybean remains the most important genetically modified (GM) crop with about 111 million hectares, corresponding to approximately 83% of the total soybean production [1]. One of the major production problems in soybean cultivation are weeds. To address this problem, the soybean MON89788 line was developed to allow for the use of glyphosate, the active ingredient in the herbicide Roundup®, as a weed control option. This event, also known as Roundup Ready 2 (RR2) was approved in the European Union (EU) in 2008 [2].

The rapid adoption of biotech crops has led many countries to develop regulations for their cultivation and trade based on the precautionary principle. In the EU, the Regulation No. 1829/2003 establishes that the labelling of all products consisting of, produced or containing GMO above 0.9% is mandatory. Bellow this threshold, the presence of GMO is considered adventitious or technically unavoidable so the labelling is not required. To guaranty the implementation of these regulations and respond to the increasing consumers' concerns related with the presence of GMO in food and feed, the development of new analytical

approaches is urgent. Molecular detection methods have assumed an important role, namely the PCR techniques due to specificity, sensitivity and reliability, which allow their application to highly processed foods [3]. The aim of this work was to develop qualitative PCR and real-time PCR assays to trace RR2 soybean in foodstuffs commercially in the Portuguese market.

2. MATERIALS AND METHODS

2.1. Reference materials and samples

Reference materials (RM) from the American Oil Chemists' Society (AOCS; Urbana, II, USA) were used as standards containing 0%, 0.1%, 1% and 10% of RR2 soybean. For method validation, samples of soybean flour were analysed under the USDA/GIPSA (Grain Inspection Packers and Stockyards Administration) Proficiency Program.

The analysed food samples included soybean grains/flours/pastas, cookies, drinks, desserts, tofu, meat analogues and sauces. All the solid samples were triturated/homogenised in a knife mill, and the liquid samples were centrifuged to pre-concentrate the DNA. All of them were stored at -20ºC.

2.2. DNA extraction

DNA was extracted by the Wizard method as described by Mafra et al. [3] and/or NucleoSpin food kit using 200-500 mg of sample, depending on the matrix. The quality and purity of extracted DNA was analysed by UV spectrophotometry.

2.3. Oligonucleotide primers and probes

For detection, confirmation and quantification of taxon-specific sequences, primers and probes described by Mafra et al. [3] were used. Event-specific primers targeting RR2 soybean were designed to amplify an expected fragment of 121 bp: SRR2-F (CCTGCTCCACTCTTCCTTTTGG), SRR2-R (GATGGGGATCAGATTGTCGTTTC), and SRR2-P (FAM-CTCTAGCGCTTCAATCGTGGTTATCAAG-BHQ1).

2.4. Qualitative PCR

PCR amplifications were carried out in 25 µL of total reaction volume, containing 2 µL of DNA extract (100 ng), 67 mM of Tris-HCl (pH 8.8), 16 mM of $(NH_4)_2SO_4$, 0.01% of Tween 20, 200 µM of each dNTP, 2.5 mM of $MgCl_2$, 160 nM of LE3/LE4 or 280 nM of SRR2-F/SRR2-R primers, and 1.0 U of SuperHot Taq DNA Polymerase (Genaxxon Bioscience GmbH, Ulm, Germany).

The reactions were performed in a MJ Mini (BioRad Laboratories, Hercules, CA, USA) thermal cycler. Temperature programmes were optimised for each set of primers LE3/LE4 or SRR2-F/SRR2-R, respectively: denaturation at 95 ºC for 5 min; amplification in 38 or 40 cycles at 95 ºC for 30 s, 60 ºC or 63 ºC for 3 s and 72 ºC for 30 s; and a final extension at 72 ºC for 5 min.

2.5. Real-time PCR

The amplifications by real-time PCR were performed in 20 µL of total reaction volume containing 2 µL of DNA extract (100 ng), 1x of SsoFast^{TM} Probes Supermix (BioRad Laboratories, Hercules, CA, USA), 600 nM or 400 nM for LE1/LE2 or SRR2-F/SRR2-R primers, respectively and 200 nM of each probe (LE-P or SRR-P). The assays were carried out on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (BioRad Laboratories, Hercules, CA, USA) using the following temperature conditions: 95 ºC for 5 min; 45 cycles at 95 ºC for 15 s and 65 ºC for 45 s, with collection of fluorescence signal at the end of each cycle. Data were processed and analysed using the software Bio-Rad CFX Manager 3.1. Each assay was repeated in two independent assays using three replicates for each sample and two replicates for each standard.

3. RESULTS AND DISCUSSION

3.1. DNA extraction

Two different DNA extraction protocols (Wizard and NucleoSpin) were tested to obtain amplifiable DNA from soy-derived foods. The results indicated that DNA extraction was carried out with success, as seen through the yield, purity, integrity.

3.2. Qualitative PCR

The qualitative PCR assay were successfully optimised using the RM, reaching a sensitivity reached was 0.1% targeting the new event-specific PCR assay (RR2 soybean). Cross-reactivity tests confirmed the specificity of the primers SRR2-F/SRR2-R. To avoid false positive and negative results, all DNA extractions were carried with a blank extraction and all PCR runs included positive and negative controls.

Soybean was detected 78% of a total of 90 food samples from the PCR assay targeting the soybean *lectin* gene. The positive samples were further tested by qualitative PCR targeting RR2 soybean event, which allowed identifying 7 positive samples, although some with faint bands.

3.2. Real-time PCR

For GMO quantification the double calibration curve method, which consists of the co-amplification of a specific sequence of the lectin gene and a region of the specific event, was used. The prerequisites for the evaluation of the real-time PCR systems were based on the available document of the definition of minimum performance requirements for analytical methods of GMO testing [4]. The obtained real-time PCR results were according to those requirements since the correlation coefficient (R^2) of standard curves was, generally, ≥0.98 (0.993 ± 0.004 and 0.983 ± 0.003), while PCR efficiencies were 100.4 ± 3.5% and 99.0 ± 5.2% for lectin and RR2 specific curves, respectively, indicating the adequacy of the standard curves for quantification (Table 1). The method was successfully validated using the 1% RM and participating in the USDA/GIPSA Proficiency Program since the measured trueness expressed as bias is within ±25% of the actual value, though slightly higher for S2015.4.2 flour, but still within the desirable Z-score. From the 7 positive samples to RR2 soybean

qualitative PCR, only 4 confirmed to be positive by real-time PCR, from which 3 had a RR2 contents in the range of 0.04-0.13%, while one was 2.89%.

Table 1. Quantitative real-time PCR results

Analysis		Actual RR soybean (%)	Lectin Gene[a]		RR Soybean Event[a]		Estimated RR soybean (%)	Bias[c] (%)
			Ct[b]	DNA (ng)	Ct	DNA (ng)		
Validation	S.2015.4.1[d]	0	-	-	-	-	-	-
	S.2015.4.2[d]	0.66	25.85 ± 0.37	129.0 ± 34.1	33.07 ± 0.37	0.697 ± 0.18	0.48	-27.3
	RM[e]	1.0	25.61 ±0.23	104.0 ±15.5	31.39 ±0.07	1.210 ±0.05	1.16	16
Unknow samples	1		29.87 ± 0.13	6.49 ± 0.06	39.21 ± 0.44	0.009 ± 0.003	0.13	
	2		25.79 ± 0.09	91.8 ± 5.60	36.0 ± 0.25	0.05 ± 0.009	0.06	
	3		26.99 ± 0.17	49.9 ± 5.94	38.38 ± 1.54	0.021 ± 0.02	0.04	
	4		31.98 ± 0.27	1.48 ± 0.29	36.61 ± 0.68	0.060 ± 0.03	2.89	

[a]Mean ± standard deviation values; [b]Ct – cycle threshold values; [c]Bias = ((mean value-true value)/true value*100). [d]USDA/GIPSA Proficiency Program samples; [e]RM -reference material.

4. CONCLUSIONS

The present study showed that a new event-specific real-time PCR method was proposed to quantify RR2 soybean in foods. The method was successfully validated through an interlaboratorial proficiency study and applied to 90 processed food samples. Application results demonstrated the need to verify the presence of GMO in foods since from the 4 positive samples to RR2 soybean, one was much above the value for mandatory threshold for GMO labelling (0.9%).

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