TRANSGENES MONITORING IN AN INDUSTRIAL SOYBEAN OIL PROCESSING BY CONVENTIONAL AND REAL-TIME POLYMERASE CHAIN REACTION

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Abstract: In recent years a great effort has been devoted to the development of new methods for the qualitative and quantitative detection of transgenic sequences in food. Most of the developed analytical methods for GMO detection are DNA-based, since protein-based assays are not suitable for processed food. For that purpose, polimerase chain reaction (PCR) and real-time quantitative PCR have been successfully applied. Since the approval of Roundup Ready (RR) soybean in Europe, the production of soybean oil using GM seeds has been increasing. Although several reports show the possibility of DNA detection in crude vegetable oils, due to the chemical treatments and high temperatures along refining, that detection is difficult to accomplish after refining.
After a previous work of applying and comparing several DNA extraction protocols, the aim of the present work was to detect soybean DNA along the industrial processing of soybean oil extraction and refining. The Nucleospin® food kit was used for the DNA extraction from oil samples. The detection of lectin gene by conventional PCR was succeeded in all steps of refining process (crude, neutralized, washed, bleached and deodorised oil). The amplification by real-time PCR using TaqMan probes confirmed the presence of soybean DNA in all the stages along the oil refining. The detection of RR soybean was observed in all the steps along the industrial oil extraction, until the crude oil, confirming the use of GM seeds. That was also obtained in the final refined oil, but not after washing and bleaching, which was consistent with the low DNA yields in those extracts, probably due to instability of those samples. These findings were never reported and represent a great achievement when considering the detection GMO in vegetable oils.

1. INTRODUCTION

In the last decade genetically modified foodstuffs have been widely spread into human and animal food chain. In 2008, the most important genetically modified crop was soybean (*Glycine max*), representing almost 53% of the world's GM planted area [1]. Since the authorization GMO in foods, a great effort has been devoted to the development of new methods for the qualitative and quantitative detection of transgenic sequences in food. The European Union (EU) has elaborated legislation for GM food control, which establishes both the legal basis for the approval procedure of GMO and the post market traceability and labelling requirements for GMO and GMO-derived food and feeds (Regulations (EC) No. 1829/2003, 1830/2003).
Most of the developed analytical methods for GMO detection are DNA-based, since protein-based assays are not suitable for highly processed food. Polymerase chain reaction (PCR) and real-time PCR-based methods have been generally accepted for regulatory compliance.

When dealing with surveillance testing of GMO in highly processed food, researchers must face with major analytical challenges. In vegetable oils, the refining process affects the presence and the integrity of the DNA in the final product, due to the steps involved in this procedure. The physical and chemical processes, such as heat treatments, variation in the pH, nuclease activity, steeping and ensilage may lead to random cleavage of genomic DNA. Regarding crude soybean oil, the extraction of amplifiable DNA has been already demonstrated, however, after the refining process that is extremely difficult to obtain [2].
The aim of this work was to detect soybean DNA along the main processes of industrial soybean oil production: extraction and refining.

2. MATERIALS AND METHODS

2.1. Soybean samples

The samples were collected along the main processes of industrial soybean oil production: extraction and refining processes (Figure 1).

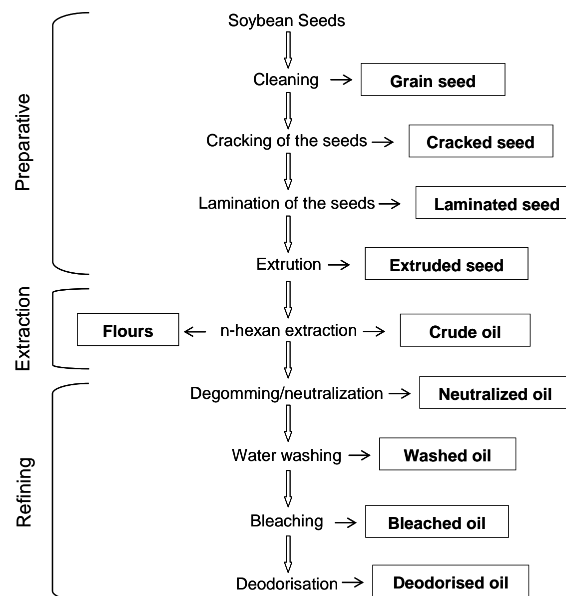

Figure 1 – Sampling from the soybean oil production

2.2. DNA extraction

The DNA extraction protocols were based on previously developed methods, namely, Wizard and CTAB [3], for solid samples. Oil samples corresponding to crude and refining steps were extracted with Nucleospin® food kit (Macherey-Nagel, Düren, Germany). Prior the Nucleospin® food kit extraction, all oil samples were pre-concentrated by centrifugation of 200 g of oil, for 30 minutes at 18000 x *g* and 4ºC.

2.3. Qualitative PCR

The DNA extracts obtained from flour samples were evaluated for their amplifiability by polymerase chain reaction (PCR) targeting the lectin gene as a marker for soybean using the conditions on Tables 1 and 2, with the primers GM03 (GCC CTC TAC TCC ACC CCC ATC C) and GM04 (GCC CAT CTGCAA GCC TTT TTG TG). The detection of sequences from

the transgenic RR soybean event was performed the specific primers GM07 (ATC CCA CTA TCC TTC GCA AGA) and GM08 (TGG GGT TTA TGG AAA TTG GAA).
The DNA extracts from oil samples were tested for the same purpose by PCR, using the primers LE1 (CCA AGC AAT GGC TAC TTC AAA G) and LE2 (TGA GTT TGC CTT GCT GGT CAG T) for lectin gene detection and the primers RRS-3J1 (GCA TCT ACA TAT AGC TTC TCG TTG) and RRS-3J3 (AAC TTCTCG ACG ATG GCC G) for event specific detection. The PCR conditions for these reactions are described on Tables 1 and 2. The amplified fragments were analysed by electrophoresis in a 2.0% agarose gel carried out in a TAE buffer (40 mM Tris-acetate, 1 mM EDTA) for 60 min at 120 V, stained with ethidium bromide (0.4 µg/mL for 5 min) and distained in distilled water for 30 min. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital Science™ DC120 (Rochester, NY, USA)

Table 1 – PCR components.

	Flours		Oils	
Component	**Lectin**	**RR**	**Lectin**	**RR**
Ultrapure water (µL)	15.05	13.8	11.8	12.3
Buffer (10 x) (µL)	2.5	2,5	2.5	2,5
dNTPs (2.5 mM each)	2	2	2	2
Taq. Polimerase (5U/µL) (µL)	0.2	0.2	0.2	0.2
$MgCl_2$ (25 mM) (µL)	1.25	2	2	2
Primers (10 mM each) (µL)	1	1.25	1.25	1
DNA extract (µL)	2	2	4	4
Total reaction volume (µL)	25	25	25	25

Table 2 – PCR amplification conditions.

	Lectin				RR			
Primers	**LE1/LE2**		**GM03/GM04**		**GM07/GM08**		**RRS-3J1/RRS-3J3**	
Steps	Temp.	Time	Temp.	Time	Temp.	Time	Temp.	Time
Denaturation	94ºC	4 min	95ºC	5 min	95ºC	5 min	94ºC	4 min
Amplification	94ºC	30 s	95ºC	30 s	95ºC	30 s	94ºC	30 s
	60ºC	30 s	65ºC	30 s	55ºC	30 s	60ºC	30 s
	72ºC	30 s	72ºC	1 min	72ºC	1 min	72ºC	30 s
Cycles	35		37		35		38	
Final extension	72ºC	4 min	72ºC	5 min	72ºC	5 min	72ºC	4 min

2.4. Quantitative PCR

Real-time PCR assays using TaqMan™ technology [4] were performed on a fluorometric thermal cycler iCycler iQ™ Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA, USA), using the primer pairs Lec-F/Lec-R and RRS-F/RRS-R and the probes Lec-TMP and RRS-TMP, respectively for lectin gene and RR soybean. PCR components and conditions were as described by ISO 21570 [4], using iQ™ SuperMix (Bio-Rad Laboratories, Hercules, CA, USA). All the reactions were performed in a total volume of 20 µL using 2 µL of template DNA in solid samples and 4 µL in oil samples. For quantitative analysis, standard curves were prepared using reference materials from IRMM (Fluka Chemie GmbH; Sigma-Aldrich; Steinheim) serially diluted (1/3).
Data was collected and analysed using iCycler iQ™ Real-Time Detection System software 3.0.1 version (Bio-Rad Laboratories, Hercules, CA, USA).

3. RESULTS AND DISCUSSION

3.1. Qualitative PCR amplification

The results for PCR assays for DNA extractions of soybean flours regarding the preparative steps for oil extraction and the crude oil, demonstrated that both CTAB and Wizard methods, were able to extract amplifiable DNA (Figure 2). The raw material provided was defined, by the oil producer, as GM soybean seeds. According to this information, 2 samples of conventional soybean seeds were also included in this study for comparative purposes. All the samples were successfully amplified for the lectin gene as expected, since all were derived of soybean seeds (Figure 2A).

In order to determine the presence of GM material, a PCR using specific primers for specific RR construction was performed to these samples. It was also possible to confirm that all the samples obtained from the soybean oil production were GM (Figure 2B). However, the PCR results evidenced that the conventional seeds tested also positively for RR soybean. This finding was confirmed by triplicate assays and was not expected since these conventional seeds were labelled as non-GMO. That must be due to cross-contamination at the industrial plant.

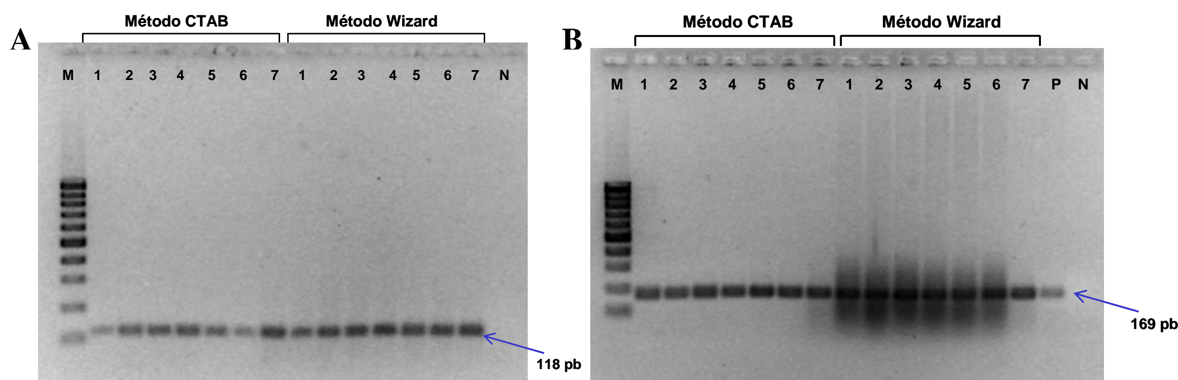

Figure 2 – PCR products of samples from preparative steps for soybean oil. A – PCR targeting soybean lectin gene. B – PCR specific for GM soybean. Lanes 1,2 - conventional soybean seeds; lane 3 - GM soybean seeds; lane - 4, cracked; lane 5 - laminated; lane 6 - extruded; lane 7 - soybean flour. N – negative control; P – positive control; M – 100 pb ladder (Bioron, Ludwigshafen, Germany).

The extracts of all refining steps (oil samples) obtained from Nucleospin® food kit, were also tested by qualitative PCR. The agarose gel electrophoresis demonstrated that all the samples from the refining process (Figure 3B) from the crude oil (lane 1) to the final deodorized oil (lane 5) amplified the lectin gene, with an expected fragment of 103 bp. A positive result was expected for the crude oil, since it was not likely to occur a high level of DNA degradation at this stage [2]. However, previous works state that after the neutralization step, it is not possible to detect amplifiable DNA [2]. The results obtained in this work evidenced that the extraction method used is determinant for a positive DNA amplification. This achievement may contribute for a new approach in the detection of DNA in vegetable oils.

For the RR soybean detection, it was used a new set of primers (RRS-3J1/RRS-3J3) conducting to a smaller fragment (106 bp) than the one previously used (GM07/GM08). The results obtained demonstrated that only the first and the final steps (lanes 1 and 5) tested positively for the presence of RR soybean (Figure 3A). Since the deodorized oil (lane 5), corresponding to the last step of the refining process, was positive for RR soybean, it was also

expected positive amplifications for the previous steps of refining. However, this fact was not observed, probably due to the instability of the intermediate samples.

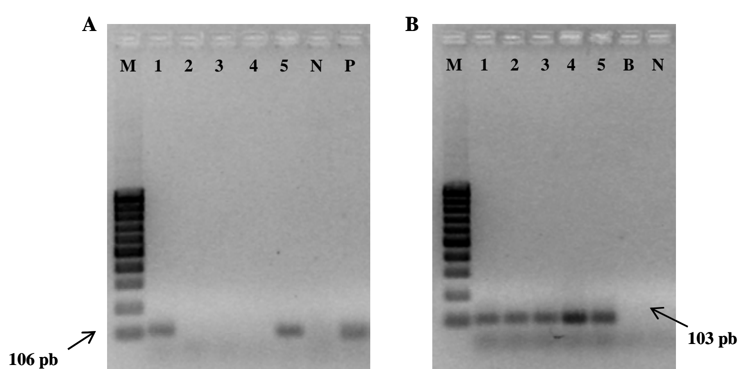

Figure 3 – PCR amplification of oil samples from refining steps. A – RR soybean detection. B – soybean lectin gene detection. Lane 1-5 – crude, neutralized, bleached and deodorized steps of refining, respectively; B – extraction blank; P – positive control (reference material of 0.1% GM soybean); N – Negative control; M – 100 pb ladder (Bioron, Ludwigshafen, Germany).

3.2. Real-time PCR amplification

In order to confirm the data, all samples were also submitted to real-time PCR analysis using hydrolysis probes. The assays were performed in parallel reactions for lectin gene and RR soybean using specific primers and probes as proposed by ISO 21570 [4], producing fragments of 81 bp and 83 bp, respectively. Each real-time PCR quantitative assay included two calibration curves, one for the lectin gene, as reference gene, and the second for the target RR soybean. The linear correlation coefficient of the standards curves (R^2) ranged between 0.991 and 0.998, and the PCR efficiencies ranged between 93.4% and 102.8%.

The samples corresponding to the preparative phases for oil extraction (solid samples) and crude oil were amplified together with the calibration curves allowing estimating the amount of DNA for lectin gene and RR soybean (Table 3). The ratio of GM DNA/lectin DNAx100 allowed estimating the proportion of RR soybean in each sample analysed. The results show a high proportion of GM material in all samples, as expected, ranging from 39.8 from 54.0%. The data also confirmed the positive result for RR soybean in the conventional soybean seed, although with a much lower value (10.1%).

Table 3 – Real-time PCR data for the solid samples corresponding pre and pos-extraction process

	Lectin			RR			
Soybean seeds	**Ct**	**DNA (ng/2µL)**	**DNA copies**	**Ct**	**DNA (ng/2µL)**	**DNA copies**	**% GM DNA**
Grain GM	27.73 ± 0.07	97.55 ± 4.88	86327.4	29.17 ± 0.11	54.63 ± 3.76	48348.1	56.0
Cracked GM	27.54 ± 0.01	111.5 ± 0.7	98672.6	29.29 ± 0.19	50.70 ± 6.41	44867.7	45.5
Laminated GM	29.14 ± 0.06	36.10 ± 1.41	31946.9	30.70 ± 0.05	19.83 ± 0.68	17551.6	54.9
Extruded GM	27.06 ± 0.07	156.5[a] ± 7.8	138495.6	28.97 ± 0.13	62.30 ± 5.14	55132.7	39.8
Flour GM	27.28 ± 0.06	134.5 ± 5.0	119026.5	28.74 ± 0.02	72.60 ± 0.85	64247.8	54.0
Conventional	28.04 ± 0.01	78.65 ± 0.21	69601.8	32.10 ± 0.02	7.91 ± 0.08	7000.0	10.1
Validation Standard 1% GMO	27.84 ± 0.00	90.30 ± 0.12	79911.5	34.32 ± 0.19	0.93 ± 0.13	823.0	1.03

a – above limit of quantification

All the 5 extracts corresponding to the refining steps (oil samples) tested also positively by real-time PCR for the lectin gene, confirming the previous results obtained in the qualitative PCR. The results contradict other authors [5], which demonstrated that only the neutralized phase presents traces of DNA, when using high quantities of neutralized oil (> 250g). It was also possible to obtain positive results for the amplification of the specific RR construction in crude, neutralized and deodorized oils. These results are in complete agreement with the previously qualitative PCR assays (Figure 3A). The RR amplification for the neutralized oil was below limit of quantification, reason because the %GM for this sample was not obtained. The samples corresponding to the washed and bleached oils gave negative results as in the previous PCR amplifications, probably due to the higher level of DNA degradation caused by the instability of the samples when stored prior to analysis. The results for crude and deodorized oils gave also high proportions of RR soybean, respectively, 83.9 and 61.4%.
These findings were never reported and represent a great achievement when considering the detection GMO in vegetable oils.

Table 4 – Real-time PCR data for the oil samples corresponding to refining process.

	Lectin			RR			
Soybean oils	**Ct**	**DNA (ng/4µL)**	**DNA copies**	**Ct**	**DNA (ng/4µL)**	**DNA copies**	**% GM DNA**
Crude	25.76 ± 0.32	9.00 ± 1.96	7967.6	26.78 ± 0.59	7.55 ± 2.98	6681.4	83.9
Neutralized	29.96 ± 0.47	0.51 ± 0.16	454.0	37.54 ± 0.66	0.0048[a] ± 0.0023	4.2	ND
Washed	31.74 ± 0.26	0.15 ± 0.03	131.4		NA		
Bleached	29.86 ± 0.04	0.53 ± 0.01	471.7		NA		
Deodorized	27.56 ± 0.05	2.30 ± 0.51	2035.4	29.59 ± 1.33	1.41 ± 1.36	1249.0	61.4
Validation Standard 1% GMO	20.85 ± 0.09	259.2 ± 16.3	229331.9	28.30 ± 0.07	2.55 ± 0.13	2256.6	0.98

a – value below limit of quantification, NA – no amplification, ND – not determined.

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