Detection of DNA During the Refining of Soybean Oil

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ABSTRACT: The isolation of DNA from foodstuffs is the first step in the detection of genetically modified organisms. Refining processes, however, have an irrevocable influence on the quality and quantity of DNA and make detection in refined oil impossible. In order to determine the most significant step in removing DNA from crude soybean oil, two refining processes were considered: chemical refining and physical refining. Although conducted on a lab scale, quality parameters showed that the refining processes were good simulations of the industrial refining. From samples drawn at various refining stages, DNA was extracted with a protocol originally developed for the extraction of DNA from lecithin. The polymerase chain reaction results prove that the protocol was sufficiently useful for extracting DNA from soybean oil. The amplified DNA revealed that degumming is the most important step in removing DNA from crude soybean oil. After degumming, DNA was concentrated in the water fraction; no DNA could be amplified in the oil fractions. During physical degumming, degradation of DNA was observed.

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KEY WORDS: Degumming, DNA, detection limit, PCR, refining, soybean oil.

The suitability of the polymerase chain reaction (PCR) technique to detect genetically modified organisms (GMO) in processed food is determined by the quantity and quality of the DNA still present in the final product and the quality of the primer. On one hand, physical and chemical treatments, such as heat treatments, change in pH, nuclease activity, refining, steeping, and ensilage may lead to random cleavage of genomic DNA (1–7). The resulting decrease in the average DNA fragment length may render the detection of the inserted DNA sequence impossible. On the other hand, the composition of foodstuffs may also have an important role in the detection of GMO. Cocoa, fat, polysaccharides, polyphenols, some proteins, and certain salts are interfering substances that can impede further analysis (5,7,8). A high purity of the extracted DNA can only be achieved with a matrix-dependent DNA-isolation. A few PCR setups for the detection of a variety of GMO have been published for different kinds of foods.

In order to examine the influence of different production steps on the quality of the DNA, samples obtained from different stages of production need to be included in DNA analysis. But systematic investigations of the effects of food processing are poor (3,6,9). In soybean oil, i.e., when crude soybean oil is simply centrifuged at $14{,}000 \times g$ for 15 min, no detectable amounts of DNA can be recovered (4), but these conditions are not commonly practiced in edible oil processing. Moreover, sample sizes of the pressed oil used were small. On the other hand, DNA traces were detected in refined rapeseed oil (10). At present, it is not unambiguously specified which refining processes remove DNA from crude oil. For this purpose, a clear understanding of refining steps as applied in industry on removing plant DNA is necessary. The aim of this study was to investigate the detectability and traceability of amplifiable DNA during the lab-scale refining of soybean oil. Two industrial refining procedures, chemical and physical refining, were considered.

Experimental procedures. Crude non-water-degummed soybean oil was provided from an industrial crushing site. The oil was obtained by extracting crushed soybeans with hexane.

Refining conditions. For chemical refining of crude oil, 800 g of oil was preheated to 70°C with stirring at 175 rpm for 10 min. Demineralized water (3 wt%) was added, and the mixture was stirred at 250 rpm. After 30 min, the oil was centrifuged for 10 min at 3000 rpm. Taking the initial FFA content into account, a calculated amount of NaOH (5 N, percent excess) was added to neutralize the FFA. During NaOH addition, the oil was mixed at 250 rpm for 1 min, followed by gentle agitation at 175 rpm for 45 min at 55°C. The oil was heated to 75°C to break any emulsion droplets formed, centrifuged at 3000 rpm for 15 min, and washed twice with 10% water. Prior to bleaching, the oil was dried under vacuum at 95°C. Acid-activated bleaching earth (1 wt%) was added and mixed with the oil for 30 min under the same conditions. After filtering, the oil was deodorized with injection of 1% steam at a temperature of 240°C and a residual pressure of 3 mbar during 45 min.

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In the physical refining process, 800 g of oil was heated to 85°C. In the degumming stage, 0.1% of a 30% citric acid solution was added and homogenized at 16,000 rpm for 1 min; 0.05% NaOH of a 20% solution and 2 wt% water were added and homogenized under the same conditions. This hydrating process was continued for 1 h at 40°C in a waterbath, while stirring at 175 rpm. The mixture was heated to 70°C and centrifuged at 3000 rpm for 15 min. Subsequently, the oil was recovered, bleached, and deodorized using the same settings as in the chemical refining, except for the deodorization, where 1.5% steam was used instead of 1%.

Evaluation of the refining process. Different process evaluation parameters were included in order to check the quality of the refining process. FFA content was determined by AOCS Official Method Ca 5a-40 (11), PV according to Method Cd 8-53 of the AOCS (11), and *p*-ansidine value by AOCS Official Method Cd 18-90 (11). To determine moisture content, AOCS Official Method Ca 2e-84 (11) was used. Phosphorus content was measured by means of inductively coupled plasma (12).

DNA isolation. DNA of the oil samples was extracted as described by Wurz *et al.* (13). As a negative control, 2 mL of water was subjected to extraction and further treated similarly to the samples. The concentration of the isolated DNA was determined with a GeneQuant pro RNA/DNA calculator (Amersham Pharmacia, Roosendaal, The Netherlands).

Oligonucleotide primers. PCR primer pair GMO3 (5′-GCC CTC TAC TCC ACC CCC ATC C-3′) and GMO4 (5′-GCC CAT CTG CAA GCC TTT TTG TG-3′) were synthesized by Life Technologies (Paisley, Scotland). This primer pair is specific for the single-copy lectin gene (4,14) and yields a PCR-product of 118 bp (14). The primers were dissolved in a TE-buffer (Tris HCl pH 8.0 + EDTA pH 8.0) to obtain 50 μM concentration.

PCR amplification. Each DNA isolation was subjected to PCR amplification. For detection, 2 μL of extracted DNA product was added to 36.6 μL bidistilled water, 5 μL of 10 × PCR-buffer (Applied Biosystems, Lennik, Belgium), 2 μL of primer GMO3 (10 pmol/μL), 2 μL of primer GMO4 (10 pmol/μL), 2 μL of deoxy nucleoside triphosphate (dNTP) (5 mM; Promega, Leiden, The Netherlands), and 0.4 μL Taq polymerase (5 U/μL; Applied Biosystems). The negative control in this PCR reaction was done with 2 μL of bidistilled water. The PCR was performed in a PE9600 PCR System of Applied Biosystems. After initial denaturation at 95°C for 3 min, 35 amplification cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 60 s were run with a final extension step of 3 min at 72°C. The PCR products were cooled to 4°C. The PCR amplification products were size-separated by electrophoresis on 2% agarose gel in TAE buffer (Calbiochem, Darmstadt, Germany), stained with ethidium bromide and visualized using a UV transilluminator system.

RESULTS AND DISCUSSION

Evaluation of the refining process. The results of analysis of FFA, phosphorus content, PV, *p*-ansidine value, and moisture level are given in Table 1. These results were compared with data found in the literature (15–20). The quantity of FFA present is a good measure of the oil quality, crude as well as purified. In this study, the FFA of the crude oil was somewhat higher than expected, but could be explained by the natural formation of FA during storage. The amount of FFA was reduced through refining to an acceptable level for industrial application. Phosphatides, including lecithin, were removed efficiently from the oil and reduced to a level of 2 ppm. Primary oxidation products, determined by the PV (0.4 meq/kg), and secondary oxidation products (aldehydes), determined by the *p*-ansidine value (3.0), were below reference levels. Finally, moisture level was low. From these results, we concluded that the two refining processes, conducted on a lab scale using 800 g starting material, yielded a refined oil of good quality. Side products were removed with high efficiency.

Detection limit of DNA. The detection limit is the lowest concentration or content in an analyte that can be detected under the experimental conditions specified in a method. The detection limit of DNA extracted from food samples is generally influenced by three factors: (i) the presence of inhibitory substances in the food matrices, (ii) the extent of DNA damage, and (iii) the average fragment length of nucleic acid. These factors are dependent on the sample itself, food-processing conditions, and the physical and chemical parameters of the extraction method (21). Before any PCR analysis was conducted, the quality of the DNA extract isolated from crude oil was examined by agarose gel electrophoresis (data not shown). The analysis showed that the DNA from the crude

TABLE 1
Quality Parameters of Crude, Degummed, and Refined Oil[a]

Sample	FFA[b] (%)	Phosphorus content (ppm)	PV (meq/kg)	*p*-Ansidine value	Moisture level (%)
Crude oil	2.04	327.8	0.8	2.8	0.50
Chemical refining					
Degummed oil	2.19	41.5	6.6	1.2	0.16
Refined oil	0.03	0.7	1.1	7.9	0.03
Physical refining					
Degummed oil	2.27	4.7	6.1	2.5	0.12
Refined oil	0.07	1.4	0.4	3.0	0.04

[a]Means of three measurements.
[b]Calculated as oleic acid.

oil was highly concentrated. The outspread signal indicated that the isolated DNA was degraded to a variable extent and that fragments of all lengths were present. The total DNA of the crude soybean oil was of sufficiently good quality for further PCR analysis. In order to determine the limit of this detection approach, DNA extracted from crude oil was diluted to several concentrations. The initial concentration of DNA, estimated by reliable spectrophotometric measurements, was 1.86 μg DNA per g crude soybean oil. After dilution, PCR of these samples was carried out.

Results (Fig. 1) demonstrate that DNA could be detected with a very clear signal until a dilution of 1:100. But even after diluting by a factor of 5000, DNA could be detected from crude soybean oil using the PCR settings as described. This proves that the DNA extraction method applied in this research makes the isolation of small amounts of DNA from soybean oil possible (in the range of pg) and indicates that DNA can be amplified from crude oil in relatively high quantities.

Detection of DNA during processing. The isolation of nucleic acids from processed foodstuffs requires special and different treatments on a case-by-case basis. Exposure to heat and pH variations are known to cause the degradation of nucleic acids (1,2,6,9). In order to determine the fate of DNA during the refining of crude soybean oil, different samples during this process were submitted to PCR analysis.

The results of the PCR test for samples taken after different chemical refining steps are shown in Figure 2. Lanes 1 and 11 are DNA-molecular weight markers. In this analysis a GeneRuler™ 100 bp DNA Ladder Plus was used (Fermentas, St. Leon Rot, Germany). This ladder allowed us to check the length of the amplicon of 118 bp, resulting from the amplification of the lectin gene when the primer pair GMO3/ GMO4 was used. Lane 10 was a positive PCR control, consisting of pure non-GM soy. Lane 8 was a negative PCR control, in which mQ water was added instead of template DNA. As can be seen in Figure 2, no contamination took place while performing the extraction. Lane 9 was a DNA extraction blank, used as negative control. They can give information about contamination of the samples by foreign soy-DNA. As no signal was observed, we concluded that the samples were free of contamination.

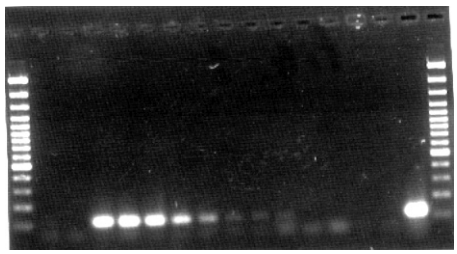

FIG. 1. Limit of detection of soybean DNA using primers GMO3/4. Lanes 1, 17: 100 bp DNA Ladder Plus; lanes 2,3: negative control; lane 4: 100% DNA from crude soybean oil; lane 5: dilution 1:5; lane 6: dilution 1:10; lane 7: dilution 1:50; lane 8: dilution 1:100; lane 9: dilution 1:500; lane 10: dilution 1:1000; lane 11: dilution 1:5000; lane 12: dilution 1:10,000; lane 13: dilution 1:50,000; lane 14: dilution 1:100,000; lane 15: extraction blank; lane 16: reference DNA from pure soy.

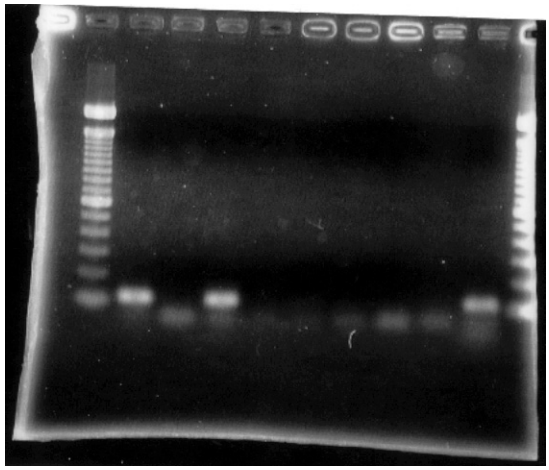

FIG. 2. Detection of soy-DNA during the chemical refining of soybean oil. Lanes 1, 11: 100 bp DNA Ladder Plus; lane 2: crude soybean oil; lane 3: degummed oil; lane 4: water fraction; lane 5: neutralized oil; lane 6: bleached oil; lane 7: deodorized oil; lane 8: negative polymerase chain reaction (PCR)-control; lane 9: extraction blank; lane 10: reference DNA from pure soy.

The crude soybean oil contained amplifiable DNA, because a clear and positive PCR signal was observed in lane 2. After degumming, no amplifiable DNA could be observed (lane 3). The observed signal was due to an excess of dNTP still present in the sample after the PCR. All or at least a major part of the DNA was found in the lecithin fraction, shown by the positive signal in lane 4. As DNA disappeared in the first stage of refining, the samples of the neutralized, bleached, and deodorized oil did not contain any amplifiable DNA. The results of intermediate samples, as well as washing waters and the bleaching earth, were negative as well (data not shown). According to these results, we concluded that the degumming process was crucial in removing DNA from soybean oil. Almost all the DNA was transferred to the lecithin water fraction, which was separated from the oil fraction by centrifugation. The quantity of DNA remaining in the samples after degumming was reduced by a factor of more than 5000 with respect to DNA.

For physical refining, similar effects were observed (Fig. 3). Again, DNA was found in the crude soybean oil (lane 2) and in the lecithin fraction (lane 4) obtained from the acid degumming. All other samples were negative (lanes 3, 5, and 6), meaning that the amount of DNA still present was below the level of detection. Compared to the results obtained in Figure 2, where the lanes of crude oil and the water fraction were of similar intensity, it can be seen that the intensities of those lanes in Figure 3 were not equal. In the latter, the signal of the water-fraction sample was less abundant than that of the crude oil sample. This could be due to the low pH, resulting from the addition of citric acid during the acid degumming. This causes degradation of the DNA. An influence of temperature on the degradation of DNA is less probable because the difference in temperatures used during chemical (70°C) and physical degumming (85°C) is rather small.

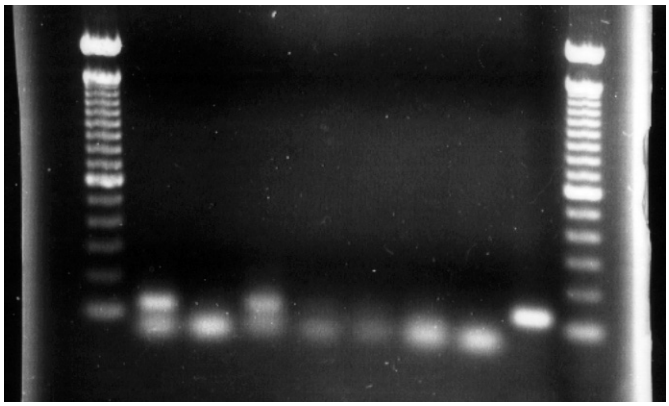

FIG. 3. Detection of soy-DNA during the physical refining of soybean oil. Lanes 1, 10: 100 bp DNA Ladder Plus; lane 2: crude soybean oil; lane 3: degummed oil; lane 4: water fraction; lane 5: bleached oil; lane 6: deodorized oil; lane 7: negative PCR-control; lane 8: extraction blank; lane 9: reference DNA from pure soy. See Figure 2 for abbreviation.

According to these results, the refining process used is a good simulation of the industrial process. It ensures an efficient removal of DNA or fragments thereof, but the total removal cannot be excluded, taking into account the limits of the detection method used. The negative results for amplification for the samples taken after the degumming step demonstrated that further analyses, i.e., detection of GMO, were best performed on the crude material. After the degumming, DNA was concentrated in the water fraction. This is an important observation, because lecithin, which can be obtained from soy oil, is used as an additive in the food industry.

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