Influence of Different Oil-Refining Parameters and Sampling Size on the Detection of Genetically Modified DNA in Soybean Oil

N. Gryson[a,b,*], K. Messens[a], and K. Dewettinck[b]

[a]Laboratory AgriFing, Department of Biotechnology, Landscape Architecture and Agriculture, Centre for Applied Research and Services, Hogeschool Gent, 9000 Gent, Belgium, and [b]Laboratory of Food Technology and Engineering, Department of Food Technology and Nutrition, Faculty of Agricultural and Applied Biological Sciences, Ghent University, 9000 Gent, Belgium

ABSTRACT: The degumming of crude soybean oil causes the removal of DNA, the presence of which is necessary for the detection of genetically modified organisms by polymerase chain reaction (PCR). In both chemical and physical refining processes, physical conditions of the refining steps can vary within a certain range. The extremities in this range may have a different influence on the quality and quantity of the extracted DNA. Therefore, the effects of degumming temperature, degumming time, and the amounts of water and/or acid added during degumming on the DNA quality and amplification were evaluated. Some parameters were shown to have a small influence on the quality of the DNA, but none affected the amplification of soy DNA extracted from the water fractions after the degumming. From the oil fractions, no DNA could be visualized. In this study of the ability of the degumming process to achieve the total removal of DNA from the oil fractions, the sample size during DNA extraction was increased significantly, and amplification of the soy lectin genes was attempted. This resulted in a positive amplification of the lectin gene. A possible relation between the size of the test portion, the residual phosphorus content of the sample, and the PCR result is suggested.

Paper no. J10623 in *JAOCS 81*, 231–234 (March 2004).

KEY WORDS: Degumming, DNA, GMO, identity preservation, PCR, real-time PCR, refining, soybean oil.

In the last decade, genetically modified foodstuffs entered the human food chain. In 2000, one-third of the soy production worldwide was genetically modified (1). Moreover, soy is one of the most important ingredients in food: 60% of all processed foods contain oil, protein, or lecithin from soy (2).

The use of genetically modified organisms (GMOs) raises protests from environmental activists and consumer organizations, especially in Europe. In October 2003, the European Union published two new Regulations regarding the admission, labeling, and traceability of GMOs (3,4). All foods produced from GMOs, irrespective of whether there is DNA or protein of GM origin in the final product, will be subjected to labeling, with a threshold of no higher than 0.9%. The European legislation demands techniques to detect GMOs in all types of foodstuffs. Owing to the diversity in food matrices, research has to be done step by step and case by case (5). Both DNA- and protein-based methods have been developed and applied for the detection of GMOs. The DNA-based polymerase chain reaction (PCR) technique is able to detect GMOs only if the quality and the quantity of the DNA present are satisfactory. In refined oil, no soy DNA can be detected; DNA is only demonstrable in the crude oil (6). Physical and chemical treatments may lead to random cleavage of genomic DNA. In this report, PCR was used to check the influence of different degumming parameters such as temperature, water content, and addition of citric acid on the detectability of DNA.

Furthermore, sampling may influence the quantity of the DNA extracted. Provided that the laboratory sample is representative of the field sample and that it has been adequately homogenized, even small aliquots of plant material are sufficient for DNA extraction, usually between 100 (7) and 350 mg (8). However, it has been shown for soybean oil that this amount is too small to generate positive amplification results (9). When a larger volume is used, DNA can be extracted from crude soybean oil (6). In this research, the effects of degumming parameters and high sampling volumes of water-degummed oil on the amplification of soy DNA were evaluated.

EXPERIMENTAL PROCEDURES

Crude soybean oil was provided from an industrial crushing site. The oil was obtained by hexane extraction of crushed soybeans.

Degumming conditions. For the chemical refining of crude oil, 400 g oil was preheated to between 50 and 70°C with gentle stirring for 10 min. Demineralized water was added (1–3%, w/w), and the mixture was gently mixed by stirring. After 30 min, the oil was centrifuged for 10 min at 920 × *g*, and the water and oil phases were stored separately at room temperature until the extraction of DNA.

In the physical refining processes, 400 g oil was heated to between 75 and 95°C. In the degumming stage, 0.05–2% (w/w) of a 30% (w/w) citric acid solution was added and mixed for 1 min. Then 0.05–2% (w/w) of a 20 wt% solution of NaOH and 2% (w/w) water were added successively with gentle mixing. All experiments were performed in duplicate.

*To whom correspondence should be addressed at Laboratory AgriFing Voskenslaan 270, 9000 Gent, Belgium. E-mail: Nicolas.Gryson@hogent.be

Evaluation of the refining process. Two parameters were determined to evaluate the quality of the refining process: the water content and the phosphorus content. To determine moisture content, AOCS Official Method Ca 2e-84 (10) was used. Phosphorus content was measured by means of the inductively coupled plasma method (ICP) (11). Measurements were done in triplicate.

DNA isolation. DNA of the oil samples was extracted in replicate with hexane and an extraction buffer based on the use of guanidine thiocyanate as described by Wurz *et al.* (12). The only adaption made was in the sample volume: Instead of 2 g, a 5-g sample was taken. After extraction, the DNA pellets were dissolved in 50 µL of twice-distilled water. As negative controls, two 5-mL samples of water were subjected to extraction and treated similarly to the samples (extraction blanks).

Qualitative PCR. 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 used for amplification. This primer pair is specific for the single-copy lectin gene and yields a PCR product of 118 base pairs (bp) (9,13). The primers (50 µM) were prepared in a TE-buffer (10 mM Tris HCl pH 8.0 + 1 mM EDTA pH 8.0). The PCR amplification reactions, as well as the visualization of the PCR products, were performed as described earlier (6). As a negative control, 2 µL of twice-distilled water (mQ) was subjected to PCR analysis (PCR blank). DNA fragments were separated by gel electrophoresis in a 2% agarose gel with Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized using the ImageMaster VDS UV transilluminator system of Amersham (Roosendal, The Netherlands).

Quantitative real-time PCR. DNA quantification was performed with the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the GMO Quant Roundup Ready™ soy DNA quantification system according to the manufacturer's instructions (Genescan). DNA samples were diluted 10-fold in mQ water.

RESULTS AND DISCUSSION

Physical refining. The influences of the amount of citric acid added and the temperature during the degumming process on the DNA quality were evaluated. Therefore, 0.05, 0.1, and 0.2% (w/w) of citric acid were added during degumming at temperatures of 70, 80, and 90°C. After degumming, samples were taken from the water and the oil fractions and DNA was extracted. While performing these extractions, extraction blanks were included as well, consisting of mQ water. The quality and amount of the extracted DNA were examined by electrophoresis of 10 µL of the obtained extract solution on an agarose gel. From the oil fractions, no DNA could be visualized following electrophoresis and staining of the gel with ethidium bromide (data not shown). For the different water fractions, a prominent fast-moving component was evident in all of the degummed samples (Fig. 1). This indicates the degradation of DNA to a great extent, yielding mostly fragments of less than 200 bp. An important decrease in DNA content could be observed at a citric acid concentration at and above 0.1% (w/w), as the overall intensity of the smear decreased (Fig. 1). Since the results of the phosphorus analysis and the water content of the water fractions do not show any significant differences between the samples, the lower intensity of the DNA smears can only be explained as being due to an effect of the citric acid on the DNA extraction efficiency.

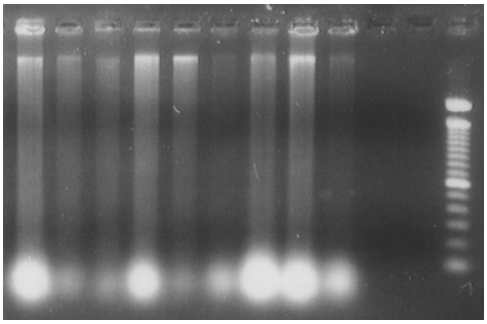

FIG. 1. Detection of soy DNA in soybean oil after physical degumming with citric acid. Lane 1: degumming at 75°C and 0.05% (w/w) acid; lane 2: 75°C and 0.1%; lane 3: 75°C and 0.2%; lane 4: 85°C and 0.05%; lane 5: 85°C and 0.1%; lane 6: 85°C and 0.2%; lane 7: 95°C and 0.05%; lane 8: 95°C and 0.1%; lane 9: 95°C and 0.2%; lanes 10 and 11: extraction blanks; lane 12: 100 base pair (bp) DNA ladder.

However, no such apparent reduction in intensity was observed for samples degummed at 95°C, compared with 75 and 85°C degumming temperatures (Fig. 1).

All of the DNA extracts from the water fractions could be amplified by PCR, independent of the degumming history. Therefore, neither a high degumming temperature nor the addition of citric acid up to 0.2% (w/w) impeded DNA amplification of the water (gum) fractions. Under all of the degumming conditions described, amplification of DNA from degummed oil remained practically unfeasible.

Chemical refining. The influence of water added (1 or 3% w/w), mixing temperature (50 or 70°C), and mixing time (15 or 30 min) during the water degumming of soybean oil on the quality and amplification of soy DNA was examined. To examine the quality of these different degumming processes, phosphorus and moisture contents of the degummed oils were determined. Through the addition of 3% (w/w) water, the phosphorus content of the oil samples after degumming was below 120 ppm, which was considered as a reference level for good degumming (14) (data not shown). When 1% (w/w) water was added, all samples had a higher phosphorus content than the 3% samples, but values were still under the reference level except for one sample (stirring at 70°C for 30 min). This sample had a remarkably higher phosphorus content, *ca.* 170 ppm. This was probably caused by more pronounced water evaporation during stirring, causing a somewhat less efficient separation of oil and water fractions through subsequent centrifugation. The higher phosphorus content had no influence, though, on the amplifiability of the DNA product (see further). All water contents of the degummed oils were slightly above the reference value of 0.1% (14,15), but this is because, in industry, the degummed oil is usually vacuum-dried (16).

To determine the influence of the different degumming parameters considered on DNA quality, DNA was extracted from the different types of degummed oil as well as from the water fractions after degumming. The quality of the extracted DNA was examined by agarose gel electrophoresis. None of the degummed oils showed residual DNA after electrophoresis (data not shown). When DNA of these different samples was amplified by PCR, no amplified DNA fragments could be detected in the oil samples after degumming. Large DNA smears were visualized for the water fractions after gel electrophoresis, comparable with the results obtained from the water fractions of the physically degummed soybean oil. Although small differences in the intensity of the signals were observed that were due to the different amounts of water added during degumming (between 1 and 3% w/w), the extracted DNA could be amplified easily for all of the water fractions. For these samples a positive amplification signal could be observed at 118 bp, resulting from the amplification of the lectin gene (Fig. 2). Oil fractions did not generate a signal at 118 bp, but a lower band could be observed, caused by the (residual) presence of deoxy nucleotide triphosphates and some primer/dimers formed. The amount of water had no influence on the amplification results. The different degumming temperatures (varying between 50 and 70°C) also did not affect the DNA amplification. Extraction blank and blank PCR, which were water samples treated the same as the samples, were negative as well, proving the good (uncontaminated) performance of the DNA extraction and PCR performance, respectively.

Sampling. The degumming step in the refining process has been shown to remove DNA from the oil. However, a higher sample volume during the isolation of DNA could make a positive PCR amplification possible. To check this assumption, DNA was isolated from water-degummed oil fractions of different sample sizes. The phosphorus content of the oil fractions used in this experiment was determined and correlation was made with the PCR result.

When the sample volume of the degummed oil was increased to 25 g instead of 5 g, with a proportional adaption of the extraction conditions, a negative PCR result was obtained for the amplification of the lectin gene. The sample size was further increased to volumes between 200 and 300 g. This sample volume was distributed into several centrifuge tubes, in which extraction buffer and hexane were added. Because of the restricted capacity of the tubes, the volume of the hexane added during the DNA extraction was reduced to 50%, and the proportion of oil to extraction buffer was maintained. At the end of the extraction procedure, the DNA pellets were gathered and pooled.

From water-degummed oil with a residual phosphorus content of 72.4 ppm, a positive PCR result was obtained when 250 g of degummed oil was extracted (Fig. 3A). For a water-degummed oil with a phosphorus content of 32.0 ppm, 250 g and even 312 g of starting material resulted in a negative PCR result, but a sample of 365 g of degummed oil gave a positive amplification signal (Fig. 3B).

These results indicate that the degumming step does not completely remove DNA from crude oil during the refining process. Raising the test portion volume can result in a positive amplification. Moreover, these results suggest that for every residual phosphorus content, a specific sample volume can be found that results in a positive amplification. In other words, a possible relationship between the residual phosphorus content of the (degummed) oil and the amplification result can be suggested.

Real-time PCR. Several procedures can be suggested to increase the sensitivity of the detection system, such as an increase of the number of PCR cycles or nested PCR. These modifications, though, might increase the risk for contamination, especially for application in routine analyses. A possible answer to this problem is the use of the Taqman® technology in a real-time PCR setup. For this purpose, the GMO Quant

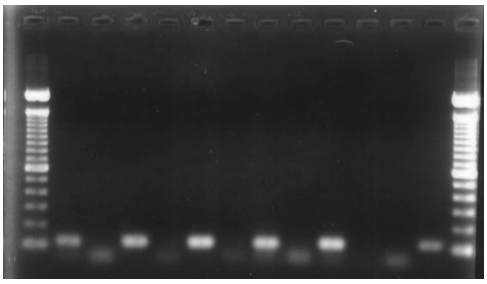

FIG. 2. Polymerase chain reaction (PCR)-based detection of soy-DNA in oil and water fractions after water degumming. Lanes 1 and 14: 100 bp DNA ladder; lane 2: crude soybean oil; lane 3: degummed at 50°C, 1 wt% water added, mixed for 30 min, oil fraction; lane 4: *ibid.*, water fraction; lane 5: 50°C, 3 wt% water, 30 min, oil fraction; lane 6: *ibid.*, water fraction; lane 7: 70°C, 1 wt% water, 30 min, oil fraction; lane 8: *ibid.*, water fraction; lane 9: 70°C, 3 wt% water, 30 min, oil fraction; lane 10: *ibid.*, water fraction; lane 11: extraction blank; lane 12: blank PCR; lane 13: reference soy DNA as template.

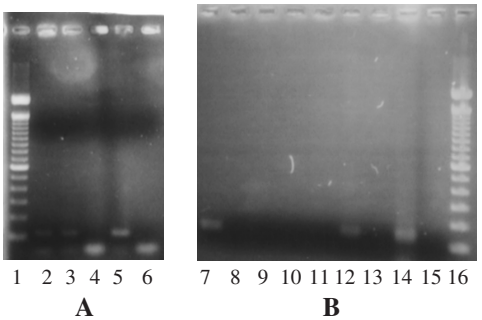

FIG. 3. Detection of soy DNA in degummed soybean oil. (A) Degummed oil with phosphorus content of 72.4 ppm. Lane 1: 100 bp DNA ladder; lane 2: crude soybean oil (75 g); lane 3: degummed sample, 250 g; lane 4: extraction blank; lane 5: reference pure soy DNA template; lane 6: blank PCR. (B) Degummed oil with phosphorus content of 32.0 ppm. Lane 7: reference pure soy DNA template; lane 8: degummed sample, 250 g; lane 9: extraction blank; lane 10: degummed sample, 312 g; lane 11: extraction blank; lane 12: degummed sample, 365 g; lane 13: extraction blank; lane 14: crude soybean oil; lane 15: blank PCR; lane 16: 100 bp DNA ladder. See Figures 1 and 2 for abbreviations.

Roundup Ready™ soy DNA quantification system of Genescan was used. With this kit, a 45-cycle PCR was run on a 1:10 diluted sample. A small signal resulting from 200 amplified copies of the soy reference gene was observed for the crude soybean oil sample with a phosphorus content of 328 ppm (test portion of 75 g). The amount of Roundup Ready soy was too low to allow a quantification of the GMO content of the sample. From a sample of degummed oil with a phosphorus content of 72 ppm (test portion of 300 g), amplification signals were below the limit of quantificaton for the soy reference DNA (= 80 copies).

The degumming process, the first step in the refining process of soybean oil, usually results in the removal of DNA from the oil phase. Degumming parameters such as temperature, water content, and amount of acids added have no significant influence on the amplifiability of the residual DNA encountered in the water phases after degumming.

We have shown here that it is possible to detect DNA by PCR in the oil phase after degumming if the DNA is extracted from a test portion with sufficiently high volume. Unfortunately, such large volumes are not feasible on a routine basis with the extraction methods described in this manuscript. As a consequence, the importance of setting up an adequate traceability system for GM soybeans and derived products cannot be underestimated. Nevertheless, the need for an analytical detection system for GMOs as a tool for their control by industry and the government will remain.

This study also indicates that there might be a correlation between the measured phosphorus content of a (degummed) oil sample and the positive amplification of the soy lectin gene by PCR.

ACKNOWLEDGMENTS

We thank Cargill (Belgium) for the supply of crude, not water-degummed soybean oil and De Smet Technology & Services N.V. (Belgium) for the water-degummed soybean oil. This work was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT-HOBU-00227)

REFERENCES

1. James, C., Global Status of Commercialized Transgenic Crops: 2000, *ISAAA Briefs No. 21*, International Service for the Acquisition of Agri-biotech Applications, Ithaca, New York, 2000.
2. Gachet, E., G.G. Martin, F. Vigneau, and G. Meer, Detection of Genetically Modified Organisms (GMOs) by PCR: A Brief Review of Methodologies Available, *Trends Food Sci. Technol. 9*:380–388 (1999).
3. Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on Genetically Modified Food and Feed, *Official J. L 268* (October 18):1–23 (2003).
4. Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of 22 September 2003 Concerning the Traceability and Labelling of Genetically Modified Organisms and the Traceability of Food and Feed Products Produced from Genetically Modified Organisms and Amending Directive 2001/18/EC, *Ibid.* (October 18):24–28 (2003).
5. Messens, K., N. Gryson, K. Supli, I. Taverniers, I. Theuns, and K. Dewettinck, Detection of Genetically Modified Organisms (GMOs) in the Food Chain: An Overview of the Currently Available Methods, *Cerevisia 25*:98–101 (2001).
6. Gryson, N., F. Ronsse, K. Messens, M. De Loose, T. Verleyen, and K. Dewettinck, Detection of DNA During the Refining of Soybean Oil, *J. Am. Oil Chem. Soc. 79*:171–174 (2002).
7. Pietsch, K., H.U. Waiblinger, P. Brodmann, and A. Wurz, Screeningsverfahren zur Identifizierung "gentechnisch veränderter" pflanzlicher Lebensmittel, *Dt. Lebensm. Rundsch. 93*:35–38 (1997).
8. Zimmerman, A., J. Lüthy, and U. Pauli, Quantitative and Qualitative Evaluation of Nine Different Extraction Methods for Nucleic Acids on Soya Bean Food Samples. *Z. Lebensm. Unters. Forsch. 207*:81–90 (1998).
9. Pauli, U., M. Liniger, and A. Zimmerman, Detection of DNA in Soybean Oil, *Ibid. 207*:264–267 (1998).
10. *Official Methods and Recommended Practices of the American Oil Chemists' Society*, 4th edn., American Oil Chemists' Society, Champaign, 1989.
11. Dijkstra, A.J., and D. Meert, Determination of Trace Elements in Oils by Plasma Emission Spectroscopy, *J. Am. Oil Chem. Soc. 59*:199–204 (1982).
12. Wurz, A., H. Rüggeberg, P. Brodmann, H.U. Waiblinger, and K. Pietsch, DNA-Extraktionsmethode für den Nachweis gentechnisch veränderter Soja in Sojalecithin, *Dt. Lebensm. Rundsch. 94*:159–161 (1998).
13. Meyer, R., F. Chardonnens, P. Hübner, and J. Lüthy, Polymerase Chain Reaction (PCR) in the Quality and Safety Assurance of Food: Detection of Soya in Processed Meat Products, *Z. Lebensm. Unters. Forsch. 203*:339–344 (1996).
14. Carr, R.A., Refining and Degumming Systems for Edible Fats and Oils, *J. Am. Oil Chem. Soc. 55*:765–777 (1978).
15. Kellens, M., Current Development in Oil Refining Technology, *Lipidex 1997*, Antwerp, 1999.
16. Dijkstra, A.J., Degumming, Refining, Washing and Drying Fats and Oils, in *Proceedings of the World Conference on Oilseed Technology and Utilization*, edited by T.H. Applewhite, AOCS Press, Champaign, 1993, pp. 138–151.

[Received April 29, 2003; accepted December 29, 2003]