Food Bioscience 11 (2015) 43–47

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/fbio

Persistence of transgenic genes and proteins during soybean food processing

Susana González-Morales[a], Marisol Cruz-Requena[a], Arturo Rodríguez-Vidal[a], Cristóbal Noé Aguilar-González[a], Óscar Noé Rebolloso-Padilla[b], Raúl Rodríguez-Herrera[a,*]

[a]Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Blvd. V. Carranza and Ing. José Cárdenas V. s/n. Col. República Ote, C.P. 25280 Saltillo, Coahuila, Mexico

[b]Department of Animal Production, Universidad Autonoma Agraria Antonio Narro, Calzada Antonio Narro No. 1923, Buenavista, C.P. 25315 Saltillo, Coahuila, Mexico

ARTICLE INFO

Article history:
Received 2 August 2014
Received in revised form
2 April 2015
Accepted 6 April 2015
Available online 16 April 2015

Keywords:
Soybean foods
*cry*1A
epsps
CAMV 35S promoter
Protein
DNA

ABSTRACT

The soybean is a typical legume which is used to elaborate several foods around the world. Ten imported soybean seed samples were collected and planted to identify possible genetic modifications. DNA was isolated from 20 days old seedlings. After that, PCR was carried out using the 35S, RR and *cry*1AB/1AS primer pairs. Nine soybean samples were identified as genetically modified. These soybeans were used to prepare six different soybean foods. During food processing, critical steps were identified; such as drastic changes in temperature and pH. A sample was taken from these critical points and from the final product for DNA extraction and PCR amplification. In most of the samples the presence of the CaMV 35S promoter and the gene *cry*1A was identified. In addition, presence of transgenic proteins was evaluated using ELISA-DAS assay. Presence of CP4 *EPSPS* proteins was detected in most of the studied soybean food samples except in yogurt and tofu. No *cry*1AB/1AC proteins were identified in any of the samples tested.

1. Introduction

During the last 20 years, development and commercial production of genetically modified (GM) crops has emerged. The GM crops have earned acceptance by farmers; that acceptance is seen in the increase in the soil used for GM crops cultivation, which has increased from 2 million hectares in 1996 to 134 million hectares in 2009 around the world (Clive, 2010). In 1994, Calgene released the first GM tomato crop utilized for human consumption, modified to have a greater shelf life (Uzogara, 2000).

Despite the agronomic advantages of GM crops, consumers have not totally accepted the products from these crops, because the suspicion that allergic problems might arise as a consequence of consumption and the lack of worldwide regulation on these crops. To assess the allergenicity of GM food, more research, including a selection of controlled sample materials and immunoassays of qualified sera, is needed (Hye-Yung, Soo-Young, Kyung-Eun, Myung-Hyun, & Kyu-Earn, 2005). In addition, the problem of contamination of non-GM foods with GM crops has made different countries to restrict the import of products made using these crops and

*Corresponding author. Tel.: +52 844 4161238, 4169213; fax: +52 844 4390511.
E-mail address: rrh961@hotmail.com (R. Rodríguez-Herrera).

http://dx.doi.org/10.1016/j.fbio.2015.04.002
2212-4292/

prohibit the use of GM crops as an ingredient or the need for specific food labeling indicating the use of GM crops (Sasson, 2000). Analysis of food products consisting of, or produced from, GM organisms is required to verify compliance with labeling legislation and to detect any unauthorized transgenic crops (Branquinho, Ferreira, & Carderelli-Leite, 2010).

Mexico imports soybean from different countries. Soybeans are utilized for creation of food and feed. During 2009, more than 28 million hectares of the land cultivated with soybean in USA was planted with different GM varieties (Clive, 2010). A high percentage of soybeans is genetically modified for traits like insect resistance and herbicide tolerance which are based on the *cry* and *epsps* genes respectively (Rincon, Ruiz, & Serrato, 1999). Although some studies have been performed to detect the presence of GMO residues in raw material and food products (Abdullha, Radu, Hassan, & Hashim, 2006; Taški-Adjuković et al., 2009; Nikolić, Taški-Adjuković, Jevtić, & Marinković, 2009; Dinon, Tremi, Sousa-de-Mello, & Maisonnave-Arisi, 2010), at present there is a lack of knowledge about the persistence of GM genes and proteins during traditional soybean processing, and a need to determine in which processing step the GMO DNA fragment or protein is degraded. Current methodologies for the analysis of genetically modified organisms are focused on either one of two targets, the transgenic DNA inserted or the novel protein expressed in a GM product (Miraglia et al., 2004). The objectives of the present study were to make six different foods with transgenic soybeans and determine persistence of transgenic genes and proteins during food processing steps and in final product.

2. Materials and methods

Soybean seed samples were taken from lots imported to Mexico through Laredo City and sown in polystyrene boxes packed with agricultural soil. Subsequently the boxes were maintained in field conditions and irrigated once every 2 days. Twenty days after planting, leaves of seedling were cut. Leaves and food samples were treated as follows: 200 mg of each sample was ground in liquid nitrogen and crushed. DNA was extracted by the method reported by Graham, Mayers, and Henry (1995). The quality of DNA was determined using agarose gel (1% w/v) electrophoresis (95 V, 40 min) in TAE buffer (Tris-Acid Acetic-EDTA) 0.5 × with 0.5 μl/mL of ethidium bromide. PCR was performed in a final concentration 25 μL with the following reagent concentrations: genomic DNA (150 ng/μL), PCR buffer (1 ×), $MgCl_2$ (3.5 mM) dNTPs (0.4 mM) and Invitrogen® Taq DNA polymerase (0.15 U/μL). PCR protocol was performed as follow; pre-incubation step at 95 °C for 5 min; 35 cycles consisting of dsDNA denaturation at 94 °C for 1 min; primer annealing at 60 °C for all *cry*'s primers, 35S and *epsps* primer pairs for 1 min and primer extension at 72 °C for 1 min. Final elongation was performed at 72 °C for 5 min. The 35S primer pair (35S1 5′-GCT CCT ACA AAT GCC ATC A-3′ and 35S2 5′-GAT AGT GGG ATT GTG CGT CA-3′) was used to identify of CaMV (Cauliflower Mosaic Virus) 35S promoter; the RR primer pair (RR01 5′-TGG CGC CCA AAG CTT GCA TGG C-3′ and RR04 5′-CCC CAA GTT CCT AAA TCT TCA AGT-3′) was used to identify the *epsps* gene, and the *cry*1AB/1AS primer pair (*cry*1AB 5′-ACC ATC AAC AGC CGC TAC AAC GAC C-3′ and *cry*1AS 5′-TGG GGA ACA GGC TCA CGA TGT CCA G-3′) was used to identify the *cry*1A gene. The PCR products were 238, 356 and 184 bp for 35S, *epsps* and *cry* primers respectively. Purified and desalted oligonucleotide primers were synthesized at Invitrogen®, before respectively diluted to a final concentration of 10 μM with double distilled water and stored at −20 °C, until further use.

All nine soybean samples identified as genetically modified were mixed and used to make six different soybean foods: tofu, soy milk, yogurt, sausages, flour and soy sprouts. During food processing, critical steps involving drastic changes of temperature and pH were identified. Following were some of the critical steps involved, soy milk (before soak, before boiling and pasteurization), yogurt (inoculation and incubation), tofu (before boiling and milk with lemon), soy sprouts (humid seeds, start of germination, and on germination at 3rd and 9th day) and soy flour (first grinding, second grinding and after dried). In the case of sausages, there was no critical step as only ingredients were mixed. A sample was taken from every critical step and also from the final product for DNA extraction and PCR amplification.

For ELISA-DAS test (Enzyme Linked Immunosorbent Assay-Double Antibody Sandwich), PathoScreen Kit of Agdia® was used to detect the GM proteins CP4 *EPSPS* (Agdia, Catalog PSP 74000 1-7) and *cry*1AB/1AC (Agdia, Catalog PSP 06200 1-5). For these tests the samples from processed foods and from each critical points were analyzed twice. The results of each sample were done in a visual form; in addition, its optical density was determined in an ELISA plate reader from Dynatech Laboratories at 630 nm.

3. Results and discussion

3.1. *Identification of genetically modified soybean*

The optimum annealing temperature for primers RR01 and RR02 which were used for amplification of part of the *epsps* gene, was determined by testing different temperatures among 50 and 62 °C; the best annealing temperature was 62 °C. The primer pair cry1AB and cry1AS which was used to identify the *cry*1A gene, also in this case a range of annealing temperatures among 55–62 °C was tested, the optimum annealing temperature for the cry primer pair was 60 °C. Results showed that the pair of primers amplified a segment of the *epsps* gene of 356 bp; and for the *cry* primer pair, the amplified segment had a size of 184 bp. Identification of the *epsps* and *cry*1A genes and CaMV 35S promoter was confirmed with sequencing of the fragment. The size of the PCR product plays a key role in the detection and quantification of GM organisms in processed foods (Yoshimura et al., 2005).

After DNA extraction and amplification, 90% of the soybean samples tested were GM and only 10% of the soybean samples were not transgenic. The *epsps* gene was detected in 80% of the tested samples, while *cry*1A gene was identified only in 40% of the imported soybean samples. Further it was observed that 40% of the imported soybean samples contained two or more genetically modified genes.

3.2. *Persistence of CaMV 35S promoter, epsps and cry1A genes during soybean food processing*

All nine soybean samples identified as GM were mixed and six different soybean foods, such as tofu, soy milk, yogurt, sausages, flour and soy sprouts were prepared. The presence of the CaMV 35S promoter was identified in yogurt, soy sprouts and flour. In the case of yogurt, in the samples taken during inoculation and incubation was detected the presence of CaMV 35S promoter; also it was observed at the start of the germination and on 3rd day of germination in soy sprouts. In soybean flour the presence of CaMV 35S promoter at all steps of processing was recorded, i.e., at first grinding, second grinding and after dried (Figs. 1 and 2). The range of temperatures to make these products was from 23 to 80 °C and the pH was in the range of 4.91 to 7.29.

The *cry*1A gene was detected in the soybean milk, yogurt and soy sprouts at the following critical points, *viz*., soaking (soy milk), boiling (soy milk), pasteurization (soy milk), inoculation (yogurt), and humid grain and at start of germination (soy sprouts) (Fig. 3). The range of temperatures to make these foods was from 21 to 94 °C and the pH was in the range of 6.56 to 9.27. Although some studies have been performed to detect the presence of GMO residues in raw material and food products (Abdullha et al., 2006; Taški-Adjuković et al., 2009; Nikolić et al., 2009; Dinon et al., 2010), in this study, we report the critical food processing steps which could affect persistence of GMO residues in the final product. The *epsps* gene was not detected in any of the critical steps and in the final food processed using GM soybeans. Difficulties encountered in the determination of GM% in processed foods from soybean have been previously reported (Yoshimura et al., 2005).

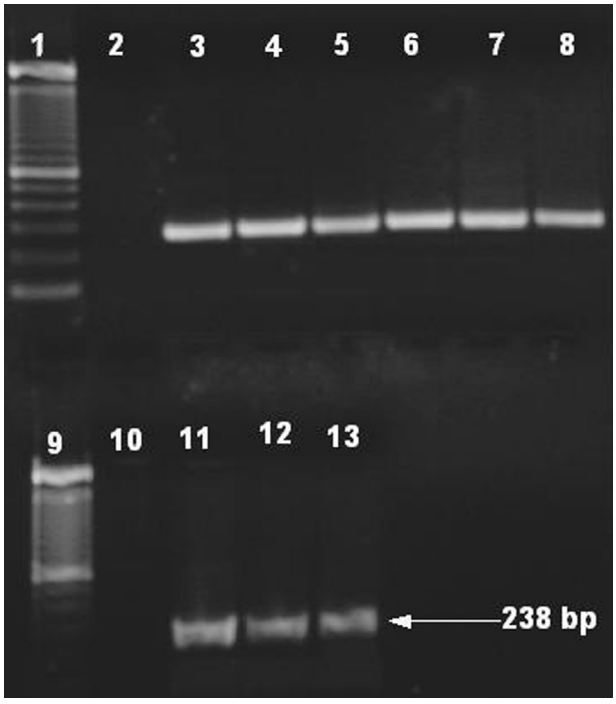

Fig. 1 – PCR amplification of CaMV 35S promoter in samples from critical steps and in final product. Annealing temperature was 60 °C, lane 1 and 9: 100 bp DNA ladder, lanes 2 and 10: negative control, lanes 3–7: critical steps of soy milk (before boiling, pasteurization and final product), and yogurt (inoculation and incubation), lanes 8, 11–13: critical steps of yogurt (final product), tofu (milk with lemon and final product), and sausages (final product).

3.3. *Identification of CP4 EPSPS and cry1AB/1AS proteins*

Presence of transgenic proteins was evaluated using PathoScreen ELISA-DAS kit. The CP4 *EPSPS* proteins have a molecular

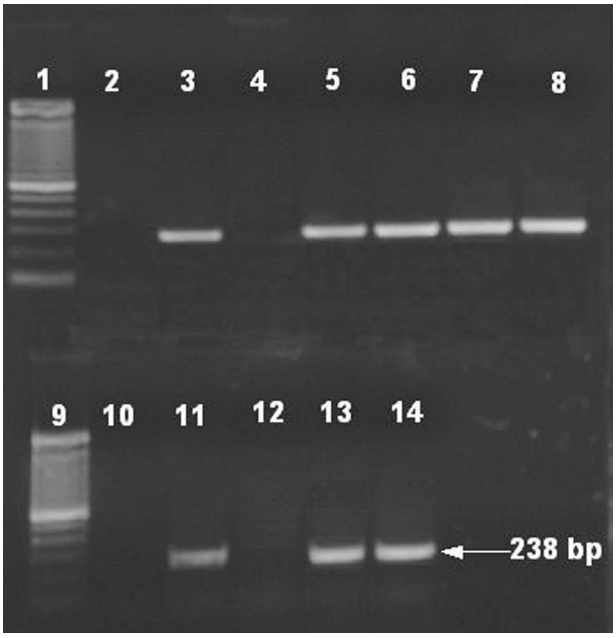

Fig. 2 – PCR amplification of CaMV 35S promoter in samples from critic steps and in final product. Annealing temperature was 60 °C, lanes 1and 9: 100 bp DNA ladder, lanes 2, 4, 10 and 12 negative control, lanes 3, 5, 6, and 7: critical steps of flour (first grinding, second grinding, after dried and final product) and lines 8, 11, 13 and 14 flour (first grinding, second grinding, after dried and final product).

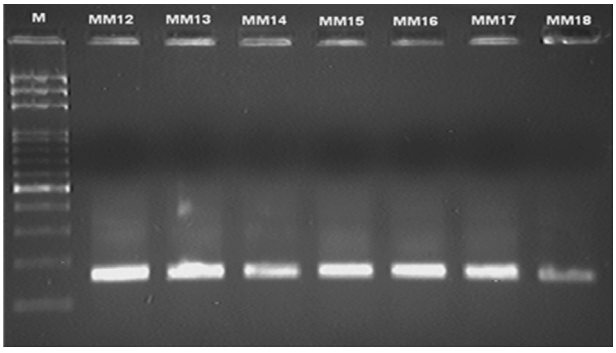

Fig. 3 – PCR amplification of *cry*1A gene (187 bp) in samples from critical steps and in final product. Annealing temperature was 60 °C, lane M: 100 bp DNA ladder, lanes MM12–15: critical steps of soy sprouts (first grinding, second grinding, after dried and final product) and lanes MM16–MM18 flour (first grinding, second grinding, and final product).

weight of 47 kDa (Van Gert, Van Biert, Bleeker-Marcelis, Peppelman, & Hessing, 1999). Elisa test revealed that 15 analyzed food samples showed values higher or equal to=0.100 of optical density in both replications (Fig. 4). To be considered as acceptable the ELISA results, means readings for negative control should be less than 0.06 and for the diluted positive control approximately 0.20. Reaction for protein detection was considered positive (presence of the transgenic protein) if optical density reading was higher or equal to three times the mean of the negative control, because negative control showed means values of optical density less than 0.03. Only were considered as positive those samples with optical densities higher than 0.1.

The presence of this protein in 15 samples of the critical steps and in final product was detected (Fig. 4). But EPSPS protein was not detected in yogurt, during the incubation step and in the final product. Similarly, CP4 EPSPS proteins were not detected in tofu samples collected before boiling, milk with lemon and in final product. The pH of the yogurt was in the range of 4.86 to 4.91, while tofu recorded the lowest pH of all samples tested and was in the range of 3.98 to 6.63. These results suggested that pH values from 3 to 6.63

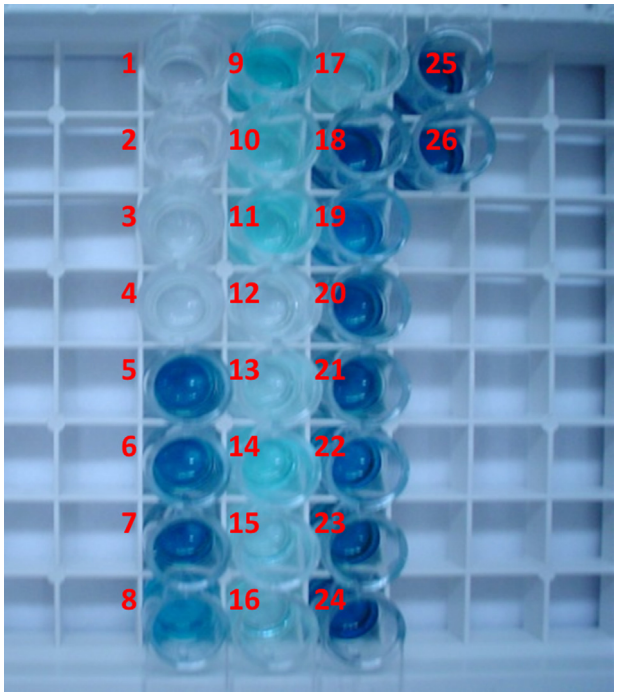

Fig. 4 – Detection of the *EPSPS* protein in soybean-based foods using ELISA-DAS test. ELISA plate's Wells: 1 and 2 blank; 3–4 negative control; 5–6 positive control; 7 soy milk (soaking); 8 soy milk (boiling); 9 soy milk (pasteurization); 10 soy milk (product); 11 yogurt (inoculation); 12 yogurt (incubation); 13 yogurt (product); 14 sausage (product); 15 tofu (before boiling); 16 tofu (milk before adding lemon); 17 tofu (product); 18 germinated (grain moistened); 19 germinated (initiation of germination); 20 germinated (germination on the third day); 21 germinated (on the ninth day germination); 22 germinated (product); 23 flour (first milling); 24 flour (second milling); 25 flour (after drying); 26 flour (product).

might have played a role in the degradation of these proteins. Nevertheless the effect of temperature on degradation of CP4 *EPSPS* proteins is low, as foods were processed at temperatures from 6 to 94 °C. The *CRY1AB/1AS* proteins have a molecular weight of 65 to 75 kDa (Gill et al., 1992). These proteins were not found in any of the critical steps or in final food product. This suggested the possibility that these proteins could have been degraded in the pH range of 3.98 to 9.27, and/ or also due to the processing temperatures from 6–94 °C. DNA methods are preferable to protein for GMO detection because of high sensitivity and stability.

The fact that 40% of the imported soybean samples contained two or more GM genes at the same time can be explained in two ways: 1) the soybean variety may have had two transgenic traits incorporated at the same or different time and in this case with insect resistance as well as tolerance to Glyphosate herbicide, and 2) during the harvest, storage, or transportation, two soybean varieties were mixed intentionally or unintentionally, thus resulting in a mixture of grains, in which one has tolerance to Glyphosate herbicide and other has insect resistance, and therefore occurred a contamination.

In 45% of the samples taken from the critical steps during food processing and in final products, the CaMV 35S promoter was detected, while only 35% of the samples showed the presence of *cry*1A gene. 45% of the samples showed more than one GM sequence, which indicate that the critical conditions during the elaboration of food processing were not sufficient to degrade these GM sequences. Transgenic monitoring in an industrial soybean processing chain is of great interest since even highly processed GMO-derived food products are covered by new European legislations (Bogani et al., 2009). PCR assays with combination of primers to CaMV 35S promoter and *cry*1A gene showed that 65% of the samples contained a GM sequence. Although items that need in the use of DNA-based detection methods include specificity, sensitivity, matrix effects, internal reference DNA, and availability of external reference materials (Miraglia et al., 2004).

The CP4 *EPSPS* protein was found in most (75%) of the soybean samples from critical steps and in final products. These proteins do not have sequences related with known allergens, and they are digested quickly in simulated mammal's digestive systems (Hoover et al., 2000). Samples with a pH value from 3.9 to 4.91 did not show the presence of CP4 *EPSPS* proteins and this suggested that pH as low as 4.9 might play a role in degradation of these proteins.

4. Conclusions

By PCR, it was possible to detect the 35S CaMV promoter and the *cry*1A gene in most of the tested soybean-based foods which suggests that some transgenic fragments resist the changes of temperature and pH during food processing. CP4 EPSPS was detected in different soybean foods, but no *cry*1AB/1AS proteins were detected. This suggested that this protein is more susceptible than CP4 EPSPS proteins to the temperature and pH employed during the processing.

Conflict of interest statement

We have no conflict of interest.

Acknowledgments

This project was financially supported by Universidad Autonoma de Coahuila (Grant: 15495/2005) and The Universidad Autonoma Agraria Antonio Narro (Grant: 13498/2006).

REFERENCES

Abdullha, T., Radu, S., Hassan, Z., & Hashim, J. K. (2006). Detection of genetically modified soy in processed food sold commercially in Malaysia by PCR-base method. *Food Chemistry*, *98*(2), 575–579.

Bogani, P., Minunni, M., Spiriti, M., Zavaglia, M., Tombelli, S., Buiatti, M., et al., (2009). Transgenes monitoring in an industrial soybean processing chain by DNA-based conventional approaches and biosensors. *Food Chemistry*, *113* (2), 658–664.

Branquinho, M. R., Ferreira, R. T. B., & Carderelli-Leite, P. (2010). Survey of compliance with labeling legislation in food containing GMOs in Brazil. *Journal of Food Composition and Analysis*, *23*(3), 220–225.

Clive J. (2010) *Global Status of Commercialized Biotech/GM Crops*. ISAAA Brief no. 42. Internet: ⟨http://www.isaaa.org/resources/publications/briefs/46/executivesummary/⟩ Accessed 08.07.14.

Dinon, A. Z., Tremi, D., Sousa-de-Mello, C., & Maisonnave-Arisi, A. C. (2010). Monitoring of GMO in Brazilian processed meat and soy-based products from 2007 to 2008. *Journal of Food Composition and Analysis*, *23*(3), 226–229.

Gill, S. S., Cowles, E. A., & Pietrantonio, P. V. (1992). The mode of action of *Bacillus thuringiensis* endotoxin. *Annual Review of Entomology*, *37*, 615–634.

Graham, H. C., Mayers, P., & Henry, R. J. (1995). A simplified method for the preparation of fungal genomic DNA for PCR an RAPD analysis. *BioTechniques*, *16*(1), 48–50.

Hoover, D., Chassy, B. M., Hall, R. L., Klee, H. J., Luchansky, J. B., Miller, H. I., et al., (2000). Human food safety evaluation of rDNA biotechnology-derived foods. *Food Technology*, 54, 56–58.

Hye-Yung, Y., Soo-Young, L., Kyung-Eun, L., Myung-Hyun, S., & Kyu-Earn, K. (2005). Genetically modified and wild soybeans: an immunologic comparison. *Allergy and Asthma Proceedings*, *26*(3), 210–216.

Miraglia, M., Berdal, K. G., Brera, C., Corbister, P., Holst-Jensen, A., Kok, E. J., et al., (2004). Detection and traceability of genetically modified organisms in the food production chain. *Food and Chemical Toxicology*, *42*(7), 1157–1180.

Nikolić, Z., Taški-Adjuković, K., Jevtić, A., & Marinković, D. (2009). Detection of GM soybean in food products by simultaneous employment of three pairs of PCR primers. *Food Research International*, *42*(3), 349–352.

Rincon, S. F., Ruiz, N. A., & Serrato, V. M. (1999). *Semillas Transgénicas* (1st ed.). Buenavista, Saltillo, Coahuila, Mexico: Editorial Universidad Autonoma Agraria Antonio Narro pp. 1–31.

Sasson, A. (2000). The contribution of biotechnologies to food production. *Elfos Scientiae*, *17*(1), 1–6.

Taški-Adjuković, K., Nikolić, Z., Vujaković, M., Milosević, M., Ingjatov, M., & Petrović, D. (2009). Detection of genetically modified organisms in processed meat products on the Serbian food market. *Meat Science*, *81*(1), 230–232.

Uzogara, S. G. (2000). The impact of genetic modification of human foods in the 21st century: a review. *Biotechnology Advances*, *18*(3), 179–206.

Van Gert, D., Van Biert, R., Bleeker-Marcelis, H., Peppelman, H., & Hessing, M. (1999). Detection methods for genetically modified crops. *Food Control*, *10*(6), 375–378.

Yoshimura, T., Kuribara, H., Matsuoka, T., Kodama, T., Iida, M., Watanabe, T., et al., (2005). Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. *Journal of Agricultural and Food Chemistry*, 53(6), 2052–2059.