

Detection of Genetically Modified Organisms in Foods by Protein- and DNA-Based Techniques: Bridging the Methods

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According to European Commission (EC) Regulation 1139/98, foods and food ingredients that are to be delivered to the final consumer in which either protein or DNA resulting from genetic modification is present, shall be subject to additional specific labeling requirements. Since 1994, genetically altered tomatoes, squash, potatoes, canola, cotton, and soy have been on the market. Recently, insect-resistant and herbicide-tolerant maize varieties have been introduced. Soy and maize are 2 of the most important vegetable crops in the world. During the past 4 years, both protein- and DNA-based methods have been developed and applied for detection of transgenic soy and maize, and their derivatives. For protein-based detection, specific monoclonal and polyclonal antibodies have been developed; for immunochemical detection, Western blot analysis and enzyme-linked immunosorbent assays are the most prominent examples. For detection of genetically modified organisms (GMOs) at the level of DNA, polymerase chain reaction-based methods are mainly used. For these reactions, highly specific primer sets are needed. This study compares the principally different methods. Specificity of methods and the possible risks of false-positive or false-negative results are considered in relation to sampling, matrix effects, and food processing procedures. In addition, quantitative aspects of protein- and DNA-based GM detection methods are presented and discussed. This is especially relevant as EC regulation 49/2000, which defines a threshold for an unintentional comingling of 1%, came into force on April 10, 2000.

Biototechnology has enabled the modification of agricultural materials in a very precise way, improving productivity and yields. The first transgenic food crops, tomatoes and canola, are now on the market, and other trans-

genic crops, such as sugar beets and sunflower, will soon follow. Transgenic cereal crops, such as rice and wheat, will probably enter the market after the year 2000.

When gene technology is applied to obtain improved functional properties, such as starch potatoes with amylopectin and without amylose and tomatoes without pectolytic enzymes, transgenic and nontransgenic crops are sold separated. However, when only agronomic properties, such as yield or herbicide resistance, have been improved, as is the case with the Roundup Ready[®] tolerant soy (Monsanto) and the Bt-resistant maize (Novartis), transgenic and nontransgenic crops are not kept apart after harvesting. In 1999, about 50% of the soy plants grown in the United States were genetically modified (1). This year the percentage will probably increase to 50–60%, and the transgenic maize varieties (Bt 176, Bt 11, T 25, and MON 810) will reach comparable numbers. In contrast, the percentage of transgenic maize in Europe in 2000 was only 0.1%.

In Europe the introduction of this genetically modified soy and maize falls under the Novel Food Regulation (EC 1139/98), which means that provisions containing transgenic soy and/or maize ingredients must be labeled (2).

In order to possibly discriminate between genetically modified and nongenetically modified crops, a series of analytical tools is a prerequisite. During the past 4 years, methods based on polymerase chain reaction (PCR) have been published for a series of transgenic crops, such as Roundup Ready soybeans (3), Bt 176 maize (4), Bt 11 maize (5), and MON 810 maize (6). At TNO Nutrition and Food Research, analytical methods have been developed for identification of materials of transgenic origin (soy and maize) in food products and of raw materials for food products. In this study, these methods are discussed in relation to specificity and sensitivity.

The introduction of the regulation concerning a threshold value per ingredient (7) requires additional quantitative detection methods. At the level of DNA detection, 2 different types of quantitative methods are available, based on competitive PCR (8) or, alternatively, on real-time PCR (9). The possible quantitation of genetically modified crops by immunochemical methods is also described (10). In this report, various types of quantitative detection methods are briefly evaluated and discussed in the light of questions related to protein expression levels, DNA copy numbers, the reference for quantitation, and the calculation of percentages at the level of protein, DNA or weight in the raw materials and in processed food materials.

Guest edited as a special report on "Method Development in Relation to Regulatory Requirements for Detection of GMOs in the Food Chain" by Elke Anklam and David Neumann.

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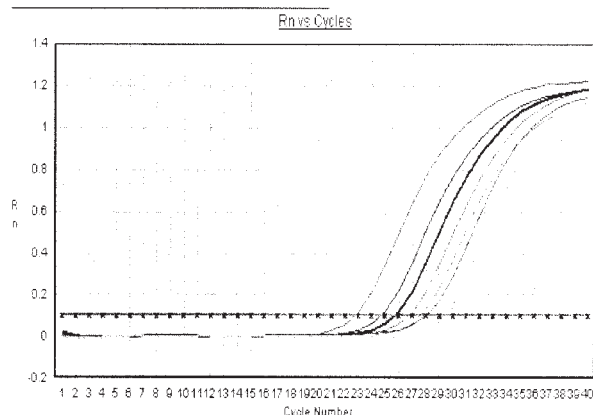


Figure 1. Quantitative PCR analysis results for serial dilution of reference sample used for calibration.

Experimental

Reference Material and Samples

Reference materials with known contents of genetically modified organisms (GMOs) were obtained from the Institute of Reference Materials and Measurements (Geel, Belgium). For soy and maize, these references were the Monsanto Roundup Ready soy and the Novartis Bt 176 maize, respectively. The contents of GMO varies from 0 to 0.1, 0.5, and 2%. By adequate mixing of these samples, standard mixtures containing 0.01 and 1% were obtained. Unknown samples, obtained from a wide variety of sources and routine analyses from customers, included raw materials (soybeans and maize kernels) and various ingredients (soy lecithin and maize starch) and final processed food products.

DNA and Protein Isolation

Before isolation of both DNA and protein various types of material, homogeneous samples were prepared by milling in a whole food machine. Depending on the type of material, between 100 and 1000 g starting material was milled to a fine powder. For DNA isolation, homogeneous samples of 150 mg were transferred into a 2 mL centrifuge tube with addition of 860 μ L extraction reagent [10mM Tris, 150mM NaCl, 2mM ethylenediaminetetraacetate (EDTA), 1% sodium dodecyl sulfate (SDS)]; 100 μ L guanidine hydrochloride solution (5mM); and 40 μ L proteinase K solution (20 mg/mL). Samples were rotated at 70°C for 1 h. (For samples containing only limited amounts of DNA, these numbers were scaled up by a factor of 10–50.) Samples were then centrifuged for 10 min at 14 000 $\times$ g. From the supernatant, 0.75 mL was transferred to a 2 mL microfuge tube and an equal volume of chloroform was added. After mixing for 10 min, phase separation was enhanced by centrifugation. From the aqueous phase, 0.5 mL was transferred to another microfuge tube, and 1 mL WizardTM resin was added to further purify the isolated DNA according

to the Promega (Madison, WI) protocol. The quality and concentration of DNA were determined spectrophotometrically at 260 and 280 nm. For protein extraction, 100 mg homogeneous sample was transferred to a 2 mL tube. The extraction is performed by using 1 mL extraction solution containing Tris (63mM); SDS (2%, w/v), pH = 6.8; 20% glycerol; and 0.01% bromophenol blue. Dithiothreitol was added before use at a final concentration of 1%.

Monoclonal Antibodies

Based on the principles of Western blot analysis (11), we developed a detection and quantitation method for the enzyme which provides the glyphosate tolerance in Roundup Ready soya plants [5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* CP4]. Because this protein was not available and in order to select for specificity, we synthesized 3 peptides corresponding to 3 parts of CP4-EPSPS. We used Swiss Protein Database analysis to derive amino acid sequences that were highly specific for the enzyme from this bacterial source.

After conjugation of these peptides to bovine serum albumin, the conjugates were used for immunization of mice to raise antibodies. Using hybridoma technology (12), we generated monoclonal antibody-producing cell lines. Purification of monoclonal antibodies was performed with immunoaffinity chromatography (Protein G).

Oligonucleotides

Because the herbicide-tolerant Bt 176 maize contains a variety of transgenic DNA, such as the *Bacillus thuringiensis* toxin gene for obtaining resistance toward the maize borer, the BAR gene for the tolerance toward the herbicide glyphosate, and the cauliflower mosaic virus (CMV) gene for regulatory purposes, different primers have been synthesized for the PCR. The oligonucleotides 35S-1 and 35S-2 are complementary to the CMV 35S promoter region. These primers were described previously and validated in a European interlaboratory trial (13, 14). The primers BAR1, BAR-2, and BAR-4 were designed for the specific detection of the BAR gene. They were used in combination with the oligonucleotide 35S-1 to amplify in the PCR reaction a unique DNA border sequence comprising parts of the BAR gene and the 35S promoter from the CMV. As a positive control for the maize DNA PCR reaction, specific primers for the alcohol dehydrogenase gene were designed.

For the Monsanto Roundup Ready soy, a comparable strategy was chosen. The oligonucleotides TN1A and TN1B are complementary to the NOS 3' terminator region. The primer TN23 was designed for specific detection of the CP4-EPSPS gene of *Agrobacterium* and was used in combination with the oligonucleotide TN-NOS3 to amplify a unique border sequence. As a positive control for the soy DNA PCR reaction, specific primers were designed for the soy lectin or heat-shock protein gene.

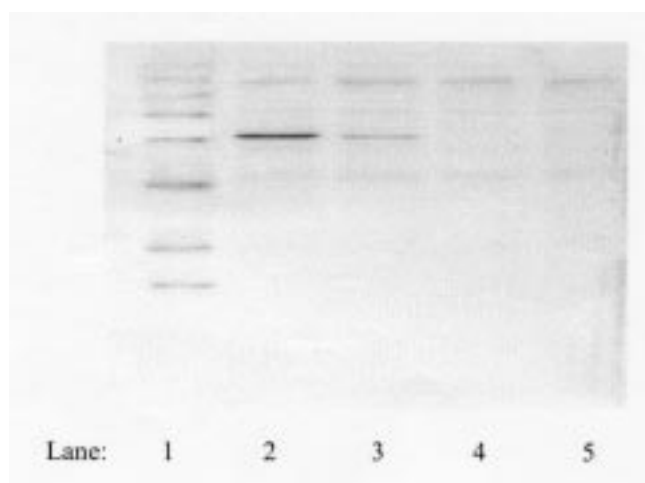


Figure 2. Western blot analysis of Monsanto Roundup Ready soy protein. Western blot analysis was performed with specific monoclonal antibodies after separation of proteins by polyacrylamide gel electrophoresis. The lanes on the gel (corresponding to those on Western blot) were loaded with equal volumes of protein extracts obtained from soy meal mixtures containing 10% (lane 2), 1% (lane 3), 0.1% lane (4), and 0% (lane 5) Monsanto Roundup Ready soy. In lane 1, a mixture of molecular weight markers was loaded.

Polymerase Chain Reaction

About 100 ng isolated DNA (5 μ L) was added to 95 μ L reaction mixture for the PCR. These amplifications were performed in 100 μ L reaction tubes containing PCR reaction buffer, 1.75mM magnesium chloride, 1 μ M of the primers, 0.2mM each of dATP, dCTP, dGTP, and dTTP, and 2.5 units of *Taq* polymerase. Amplification was performed during 40 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s) after a first denaturation at 95°C for 10 min.

When the PCR was performed with the oligonucleotides 35S-1 and BAR-2, or TN23 and TN-NOS3, 2 μ L of the final product was used for a nested PCR experiment. This nested PCR is performed under circumstances identical to those of the regular PCR, except that only 20 amplifications are performed and the primer set BAR-1 and BAR-4 (or TN1A and TN1B) is used.

Amplification products are electrophoresed on 4% nondenaturing polyacrylamide gel and made visible by staining with ethidium bromide at UV (254 nm) transillumination. Data are registered by video scanning. The expected size of amplified fragments is estimated by comparison with DNA fragments of known sizes.

Quantitative PCR

For quantitation, real-time PCR using ABI Prism SDS 7700 (Applied Biosystems) was performed. For the so-called TAQMAN technology, a third oligonucleotide labeled with a fluorescent probe (FAM) is used in combination with a usual

primer pair. As a standard procedure, quantitative PCR experiments are performed with a volume of 25 μ L containing 15 μ L TAQMAN Universal PCR master mix, including oligonucleotides and *Taq* polymerase, and 10 μ L DNA extract. Amplification is performed for 40 cycles (95°C for 9 s and 60°C for 60 s) after initial activation of Uracil–DNA glycolase and *Taq* polymerase (50°C for 2 min and 95°C for 10 min, respectively). DNA amplification increases the fluorescent signal, which is proportional to the amount of amplified DNA (Figure 1). Calibration curves of cycle threshold values vs concentrations of DNA obtained from reference samples with known concentrations of GMO are used to quantitate unknown samples.

To quantitate DNA in processed food materials, the size of fragments to be amplified should be ca 100 bp. As described here, GMO-specific PCR primers and GMO-specific probes were designed for the CMV 35S promotor region, the EPSPS gene of *Agrobacterium* CP4 as present in the Monsanto Roundup Ready soy DNA, and the BAR gene as represented in the Novartis Bt 176 maize variety. The heat-shock protein and the alcohol dehydrogenase gene were chosen for development of species-specific PCR reactions for soy and maize, respectively.

Results and Discussion

Protein Analysis

By using highly specific monoclonal antibodies, the transgenic soy protein EPSPS can be visualized with Western blot analysis. At the level of 47 kD proteins, the sensitivity of this protein method is around 1% for raw soybean meal (Figure 2). An internal validation study revealed (data not shown) that this protein analysis system is applicable for detection of transgenic Roundup Ready soy protein in raw materials and soy protein fractions. However, further processing of soy ingredients results in loss of immunochemical recognition. Western blot analysis gives additional information about the specificity of antibodies used compared with detection systems based on enzyme-linked immunosorbent assay (ELISA) techniques (10). However, as with ELISA-based methods for GMO testing, the character of Western blot analysis is predominantly qualitative because detection is based on recognition and quantitation of one specific protein. As long as no internal “housekeeping” reference protein can be quantitated, no precise calculation can be made for the percentage of transgenic material. This is especially relevant in those cases where the purity of the crop cannot be guaranteed.

Qualitative PCR Analysis

The first prerequisite for using a test to determine the presence of transgenic DNA in food ingredients or food samples is specificity. Therefore, in a first series of experiments, standard solutions of Roundup Ready soy DNA, diluted in several ratios with nongenetically modified soy DNA, were tested in PCR amplification experiments using the different available primer sets. In a typical experiment, the percentages of Roundup Ready soy DNA in mixtures with soy DNA were 1,

0.1, 0.01, and 0%. As a positive control PCR experiment, the amplification reaction was performed by using primers that specifically anneal with the soy lectin gene. For all 4 different DNA preparations, a positive signal was observed with an expected size of 120 bp (Figure 3). Because the total amount of soy DNA was comparable in these 4 reactions, no significant differences in signal intensities were expected. Besides these expected amplicons, some weak bands representing higher molecular sizes can be seen in Figure 3. These fragments possibly represent products from the first PCR reaction, although some aspecific amplification in the "nested" PCR reaction cannot be excluded. Further, the negative water control experiments is negative (Figure 3, lane 1).

Interestingly, using the primer combination TN23 and TN-NOS3 in a nested PCR experiment, a very specific PCR amplification was performed to determine the presence of the Roundup Ready DNA construct comprising the CP4-EPSPS and NOS terminator sequence. For this primer combination, specific Roundup Ready DNA amplifications (amplicons of 123 bp) had a sensitivity of 0.01% (Figure 3; panel B). In the absence of Roundup Ready DNA, no signals were found by using these primers, which indicates the specificity of this method. For maize, identical series of experiments were performed with fully comparable results (data not shown). These results indicate that this PCR analysis is very sensitive and accurate for detection of transgenic crops; however, the use of a good combination of different primer sets and the performance of adequate control experiments are prerequisites.

Figure 3; panel C shows the screening for the presence of a CMV 35S promotor sequence. In PCR screening, the sensitivity was around 0.1% for Roundup Ready soy DNA. This sensitivity is consistent with data from a collaborative trial published previously (13). In such a single-step PCR experiment, different signal intensities can be discriminated on a gel as a result of the various percentages of transgenic material. However, this diversity cannot be used for a real quantitation because the signal intensity in PCR experiments is also strongly dependent on the total amount of DNA used.

During the past 4 years, thousands of different food samples have been analyzed by these types of methods. The overall conclusion is that in nearly all cases, soy or maize DNA could be amplified in samples in which a soy or maize ingredient was present, for example in samples of biscuits, soy burgers, flavors, snacks, bread, chocolate, and ice cream. Further, of course, in the seeds, meals, and flours, as well as in derived ingredients such as lecithin and starch, DNA could be amplified. In some cases, DNA could be amplified even in soy oil and in maize syrups. Although amplification of DNA from highly processed food products may be related to the absolute amount of DNA present in the sample, profound degradation of DNA will complicate interpretation of analytical results if the housekeeping gene and the transgene are not degraded equally or if degradation resulted in DNA fragments that are below the amplifiable size, as discussed by Wiseman (15).

Finally, inhibitors (matrix effects) may influence the possibility of DNA amplification; however, this problem can be solved in most cases by an adequate balance between the

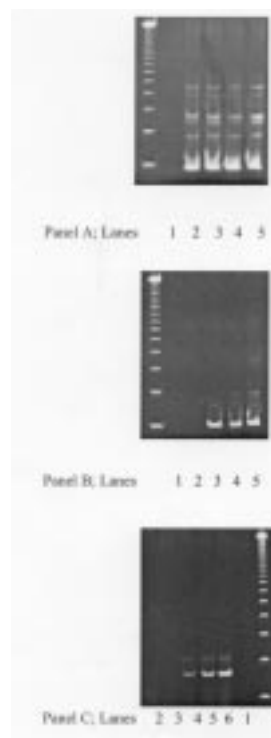


Figure 3. Video scans of PCR products after gel electrophoresis. Panel A: soy control PCR for the lectin gene; panel B: Roundup Ready soy-specific PCR; panel C: CMV 35S promotor-specific PCR. Lane 1: water control experiments; lanes 2–6: samples containing 0, 0.01, 0.1, 1, and 2% Roundup Ready soy, respectively. Molecular weight markers are multiples of 123 bp.

amount of sample used for DNA extraction and the degree of dilution of the resulting DNA extract in the PCR experiment. Of course, for each type of sample the best DNA extraction procedure should be performed. A series of various standard DNA extraction procedures has been presented by the Working Group 11 within the Technical Committee CEN/TC 275 (16).

The possibility of determining the presence of transgenic DNA is principally the same as that described for soy and maize housekeeping genes. As stated earlier, the sensitivity for detection of transgenic DNA in raw materials is around 0.01%. This sensitivity decreases proportionally with the decrease of the absolute amount of DNA in the derived ingredients and food products.

Quantitative PCR Analysis

As concluded above, there is a lack of quantitative GMO analyses of processed food materials. For example, protein-based methods are exclusively useful in the raw soy and maize materials and their corresponding meals. DNA-based PCR methods can be used for DNA amplification in all types of products, but their nature is highly qualitative. Thus, there is an urgent need for quantitative GMO analyses at the DNA level, as the defined threshold of 1%, as described in EC regulation 49/2000, became active in April 2000 (7). The introduction of real-time PCR (9) possibly allows this quantitation.

Last year we developed real-time PCR systems for various DNA targets such as the soy lectin and heat-shock protein genes, the maize alcohol dehydrogenase gene, CMV 35S promotor regions, and GMO-specific systems for various transgenic soy and maize varieties.

With respect to sensitivity and specificity, we performed the same series of experiments as for the qualitative PCR analyses. Using the validated reference materials for Monsanto Roundup Ready soy and Novartis Bt 176 maize, sensitivities as low as 0.01% were reached (Figure 1). Specificity of soy- or maize-specific PCR reactions was tested using DNA extracts from a series of crops, including wheat, rice, and barley. In none of these cases was increase of fluorescence observed in real-time PCR measurements by using primer-probe combinations designed for soy or maize.

Quantitative real-time PCR that we used in this study has not been validated yet. In spite of that, the presented technique will allow quantitation of target genes. Furthermore, in multiplex PCR experiments in which housekeeping gene DNA and transgenic target DNA are amplified and quantitated simultaneously, a calculation of the percentage of transgenic DNA at the ingredient level is possible. However, these sophisticated quantitations can be hampered by the complexity of the biological system as found in the differences in copy numbers of the same gene in various crop varieties (15). Further, as also discussed in the International Life Sciences Institute (ILSI) working group on GMO detection (15), the possibility of stacked genes might complicate the exact quantitation in accordance with the European Commission (EC) legislation.

Finally, it is concluded that for a qualitative GMO analysis, protein- and DNA-based methods can form a bridge between the raw materials and the final processed food products, thereby including their ingredients. However, real percentage quantitation of GMOs is possible only under certain restrictions such as knowledge of the variety and the copy numbers of its target gene.

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