

Qualitative and Quantitative Detection of Protein and Genetic Traits in Genetically Modified Food

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ABSTRACT

Due to the market introduction of genetically modified organisms (GMOs) in crops, foods, and ingredients, legislation worldwide came face to face with the question of the use and labeling requirements on GMO crops and their derivatives. In this review, protein- and DNA-based methods, such as enzyme-linked immunosorbent assay, western blots, and qualitative and quantitative polymerase chain reaction PCR (Q-PCR) are reviewed. Qualitative detection methods for genetically modified (GM) sequences in foods have evolved rapidly during the past years. The sensitivity of these systems is extremely high, even for processed foodstuffs. However, the availability of quantitative detection methods for GMO analysis is an important prerequisite for the introduction of threshold limits for GMOs in food. The recently introduced labeling threshold for GMOs in food ingredients by the European Union has forced official food control

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laboratories to apply quantitative PCR methods. Taking the precision of quantitative PCR detection methods into account, suitable sample plans and sample sizes for GMO analysis are discussed. As quantitative GMO detection methods measure GMO contents of samples in relation to reference material, priority must be given to international agreements and standardization on certified reference materials. The rapidly increasing number of GM foods on the market demands the development of more advanced multidetection systems, such as microarray technology. Challenges and problems arising from the inability to detect GM foods for which the modified sequence is unknown, the lengthy standardization procedures, and the need to continuously update databases comprising commercially available GM foods and the respective detection strategies are also discussed.

Key Words: GMOs; Qualitative; Quantitative detection methods

INTRODUCTION

Genetically engineered crop plantings increased 15% to 67.7 million hectares in 2003, according to a recently released report by the International Service for the Acquisition of Agri-biotech Applications (ISAAA), despite continuing consumer resistance in Europe and elsewhere (Kulkarni, 2004).

Seven million farmers in 18 countries grew bioengineered crops on 167.2 million acres in 2003, compared to 145 million acres in 2002, according to the ISAAA report. In 1996, which was the first year that genetically modified crops were commercially available, about 4.3 million acres were under biotechnology cultivation.

In India, the biotech cropping area grew 100% as a result of significant increases in biotech (Bt) cotton area. India, which planted biotech cotton for the first time in 2002, doubled its Bt cotton area to approximately 247,000 acres in 2003.

The number of countries responsible for 99% of the total biotech crop area expanded to six, up from four in 2002, according to the report. Brazil and South Africa joined the United States, Argentina, Canada, and China as the leading producers of biotech crops. China and South Africa experienced the greatest annual increases, with both countries planting one-third more biotech hectares than in 2002. The remaining top 10 countries planting more than 50,000 hectares are Australia, India, Romania, and Uruguay; another eight countries each plant up to 50,000 hectares of biotech crops.

In Europe modified foods have not gained acceptance because of consumer suspicion resulting from earlier food and environmental concerns, transparent regulatory oversight, and mistrust in government bureaucracies. All these factors have fueled debates about the environmental and public health safety issues of introduced genes (i.e., potential gene flow to other organisms, the destruction of agricultural diversity, antibiotic resistance, and gastrointestinal problems) (Gaskell et al., 1999). The European Union (EU) regulations mandate labeling of food containing GMOs (Council Regulation (EC), 1998). These regulations



established a 1% threshold for contamination of unmodified foods with GM food products (Council Regulation (EC), 2000).

Legislation in some countries makes it imperative that governments, the food industry, testing laboratories, and crop producers develop ways to accurately quantitate GMOs in crops, foods, and food ingredients to assure compliance with threshold levels of GM products. In the United States, GM plants have been approved for food use since 1992, followed by the important main staple crops like corn, rapeseed, and soya. In 1997, GM soya (Monsanto Roundup Ready) represented approximately 10% of the total soya harvest in the United States and in 2004, this has increased to almost all soya production (soya is one of the most versatile staple crops and is used as an ingredient in over 30,000 foods) (Giles, 2004).

In the United States, food safety and labeling of GM plants are regulated by the Food and Drug Administration (FDA), the United States Department of Agriculture (USDA) and the Environmental Protection Agency (EPA). These agencies are responsible for the review and evaluation of the mountains of data that are generated by rigorous and thorough testing and have found that there is no evidence that genetically engineered foods are inherently less safe than are foods derived from conventional techniques. Therefore, the United States does not believe that GM foods, as a class, require mandatory labeling.

The European Union, on the other hand, has favored a labeling approach that aims at informing consumers of the GMO origin of products. A genetically modified organism is one in which the genetic material has been altered in a way that does not occur naturally.

Genetic modifications are usually carried out by the insertion of a synthetic string of several smaller pieces of DNA from various sources into the genome of the organism to be modified. This process is called transformation. Normally, the components of the insert originate from other naturally occurring organisms. A typical insert (gene construct) is composed of three elements: (1) the promoter element functions as an on/off switch for transcribing the inserted/modified gene; (2) the inserted/modified gene that codes for a specifically selected trait; and (3) the terminator element functions as a stop signal for transcribing the inserted/ altered gene.

PRODUCTION OF GM PLANTS

Transformation of plants is achieved by the insertion of appropriate DNA into a single cell, which is then regrown into a complete organism. There are two methods generally used for the introduction of DNA into plants.

The first method of transformation is the so-called “shot-gun” approach method, which is rarely used today. The other method uses transfection with an agrobacterium, which infects the plant and thereby transfers its DNA to the plant. The simple introduction of only the gene coding for the new property, however, is not enough. In order for the gene to be expressed as a protein, other elements must also be included in the GM construct. These additional features include a promoter (or start) signal and a terminator (or stop) signal. Often a marker gene is also included allowing for selection of transformed from nontransformed cells.



The promoter serves as a start signal to switch on gene expression and production of the protein. In many of the approved plant varieties, the 35S promoter derived from the Cauliflower Mosaic Virus (CaMV) is used. The induced gene encodes for the new protein. The gene can occur naturally or can be fully synthetic. The terminator sequence is the stop signal. The terminator in many of the approved plants is derived from the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*, a common soil bacterium. This terminator is called NOS or NOS 3.

In addition, several other elements can be present in a gene construct. Usually their purpose is to control and stabilize the function of the gene, to demonstrate the presence of the construct in the GMO, or to facilitate the combination of the various elements in the construct.

EUROPEAN LABELING REGULATIONS

The detection of GM crops is necessary for several reasons, one of the most important is to comply with labeling regulations that themselves allow end consumers to make an informed choice.

On April 10, 2000, two new labeling regulations came into force: 49/2000/EC, which stipulates a 1% threshold of GM material in products otherwise derived from identity-preserved sources (e.g., organic, non-GMO grain) and 50/2000/EC, which requires labeling of flavors and additives (the latter were previously exempt from labeling) (Council Regulation (EC), 2000). A consequence of the declaration of a de minimus threshold of 1% was the need to progress from a qualitative detection of the transgenic species by using an appropriately validated screening system to more complex quantitative procedures. Following the qualitative detection of a GMO, manufacturers would be required to label the product, or, in the case of identity-preserved sources, determine whether the amount of GMOs present was above or below the 1% threshold. The implication is that the method used to quantitate the level of GMO should be as accurate as possible. The effects of the regulations are such that all aspects of the food chain require detailed analyses involving a large number of food matrices.

DETECTING GMOs

Food, of course, contains a range of other substances, such as lipids, fatty acids, and polysaccharides, in addition to DNA and protein. Some of these substances may negatively affect the assay techniques used to detect GMOs. For example, the presence of some plant polysaccharides can effectively inhibit polymerase chain reaction (PCR). In the absence of appropriate controls, this could be interpreted as a false negative.

The degree of degradation of the material to be tested is also important. If either protein or DNA is to be detected and both are too degraded to be recognized, e.g., by antibodies or primers, there is also the potential for generating a false-negative result. Strict application of appropriate test controls is necessary to avoid this situation. Likewise, the sample to be tested must be homogeneous and truly



representative of the bulk material. Therefore, each method must be validated for the food type for which it will be used.

There are two basic strategies in GM testing, namely detection of the product (e.g., a specific protein) of the modified gene or detection of the genetic material itself.

The GM products contain an additional trait encoded by an introduced gene(s), which generally produce an additional protein(s) that confers the trait of interest. Raw material (e.g., foods) derived from GM crops might thus be identified by testing for the presence of introduced DNA or by detecting expressed novel proteins encoded by the genetic material. Both qualitative (i.e., those that give a yes/no answer) and quantitative methods are available (Schreiber, 1999).

SAMPLING OF GM PRODUCTS FOR TESTING

Both sample size and sampling procedures are important issues when testing for GMOs in raw material and food ingredients if one is to avoid problems of nonhomogeneity. The sampling plan should be performed in a manner that ensures a statistically representative sample; and the sample size must be sufficient to allow adequate sensitivity, because the statistical significance achievable with a small sample size is weak (Gilbert, 1999). Given the threshold value of 1% of GM material within conventional material, laboratory samples for GMO analysis should contain a sample size of at least 10,000 to get an overall sampling error of 20%, considering that a relative sampling error of 20% is acceptable (Hübner, 2001). The optimum sampling strategy is a balance between sensitivity, cost, and confidence. It would, however, be desirable if sampling plans were coordinated on a worldwide basis [e.g., through the Codex Alimentarius Commission of the United Nations (UN)] to ensure adequacy of testing. The sampling procedure for large cargoes, such as rail cars, trucks, or ships, consists of combining increments taken from different positions to form the bulk sample and reducing this bulk sample to a laboratory sample. For the correct interpretation of the analytical report, information on the sampling procedure must be provided.

REFERENCE MATERIALS FOR GMO TESTING

Appropriate reference materials for positive and negative controls provide the basis for the validation of analytical procedures and for assessing the performance of methods and laboratories. Reference material should be independent of the analytical methods and should be focused on raw materials, or base ingredients rather than on finished foods.

Each GMO requires specific reference material. The Institute of Reference Materials and Measurements at the Joint Research Center in Geel, Belgium, offers a set limited number of reference materials, through Fluka (Buchs, Switzerland) for modified soya, corn, and maximizer maize. For international reliability of GMO testing, internationally standardized reference materials are absolutely required.



Every quantitative GMO analysis must be calibrated either with calibrants or with material that can be traced back to calibrants. If needed, a conversion factor can be published for new production series. Important characterization of the raw material used for the production of calibrants includes homogeneity, homozygosity for nonhybrid plants, and polyploidy status for hybrid plants (Trapmann et al., 2002).

DETECTION OF PROTEIN PRODUCED BY THE GENE

Immunoassay is the current method for detection and quantification of “new” proteins introduced through genetic modulation of plants. Different formats are suitable for field use as well as for well-equipped laboratories. The crucial component of an immunoassay is an antibody with high specificity and affinity for the target molecule. To meet the requirement of community legislation, assays must allow the protein to be measured at the permitted threshold level. Immunoassays can be rendered highly specific and samples often need only a simple preparation before being analyzed. Moreover, immunoassays can be used qualitatively (yes or no answer) or quantitatively over a wide range of concentrations.

For GMOs, the antigen that is used for the development of the immunoassay, is typically a purified protein. For new proteins introduced through biotechnology, the development of immunoassays is dependent on the access to the new proteins (Brett et al., 2000). These are proprietary proteins of the company who developed the crop and, thus, not commonly available for the development of methods. Access to proprietary proteins is needed to be able to raise antibodies to that new protein.

Immunological methods demand either polyclonal antibodies or monoclonal antibodies. Both polyclonal and monoclonal antibodies may require further purification steps to enhance the sensitivity and reduce background readings in assays. The specificity of the antibodies must be carefully checked to elucidate any cross-reactivity with similar substances, which might cause false positive results.

Immunoassays utilize the specific binding of the antibody to its target (i.e., the antigen or analyte) (Lipton et al., 2000). Thus, the availability of antibodies with the desired affinity and specificity is the most important factor for designing test systems. The reaction between the antigen and antibody is detected through a second antibody, preferably reacting with another epitope on the antigen. The second antibody carries a label that can be detected or can generate a detectable signal. Current food assays are almost exclusively based on the colloidal gold technique. On the basis of typical concentrations of transgenic material in plant tissue ($>10 \mu\text{g}$ per tissue), the detection limits of protein immunoassays can predict the presence of modified proteins in the range of 0.01% GMOs (Stave et al., 2004). Immunoassays with antibodies attached to a solid phase have been used in two formats: an assay in which the detector and analyte compete to bind with captured antibodies or a two-site (double antibody sandwich) assay in which the analyte is sandwiched between the capture antibody and the detector antibody.

Both western blot and enzyme-linked immunosorbent assay (ELISA) techniques have been used for the analysis of protein products of Monsanto's transgenic



Roundup Ready soybean (RRS), which is resistant to the herbicide glyphosate and contains the gene encoding *Agrobacterium* spp. strain CP4-derived 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) (Rogan et al., 1999).

Western Blot

The western blot is a highly specific test method that provides qualitative results suitable for determining whether a sample contains the target protein below or above a predetermined threshold level. It is particularly useful for the analysis of insoluble protein. The detection limits of western blots vary between 0.1 and 1%. Sensitivity is dependent not only on affinity level, but also on the level of expression of the protein in the plant (Stave; Van Duijn et al., 1999).

ELISA

The ELISA assumes more than one format: either a microwell plate (or strip) format or a coated-tube format. The antibody-coated microwells, with removable strips of 8–12 wells, are quantitative, highly sensitive, economical, provide high throughput, and are ideal for quantitative high-volume laboratory analysis, provided the protein is not denatured. The typical run time for a plate assay is 90 min, and an optical plate reader determines concentration levels in the samples. Detection limits vary between 0.8 and 1.2% (Stave et al., 2004). The antibody-coated tube format is suited for field-testing, with typical run times ranging from 15–30 min and tubes can be read either visually or by an optical tube reader; the results are qualitative. The ELISA methods can easily be automated. It offers a number of other benefits, including high throughput and quantitation of the result by inclusion of an appropriate standard curve.

The main drawback of using immunological systems to detect the protein product of the gene is that the transgenic proteins may not be expressed (or expressed only at low levels) in the part of the plant that is used in food production.

For example, the Bt toxin protein is expressed in the green leaf parts of Novartis Bt 176 maize but only in very low levels in the maize kernels.

In addition, some introduced DNA sequences are not expressed as proteins, as in the case of a transgenic tomato, where only RNA is produced to inhibit the translation of a protein that influences the ripening process.

A variation on ELISA, using strips rather than microtiter wells, led to development of lateral flow strip technology. The lateral flow format gives results in 5–10 min, is economical, more amenable to point-of-sale application, and is suitable as an initial screening method early in the food chain. These strips have been developed commercially to detect endotoxins expressed by the bacterium *Bacillus thuringiensis*, which protect against insects, as CryI(Ab) in corn plants, seeds, and grains, in addition to the CP4-EPSPS protein in soybean, canola, cotton, and sugar beet. Protein-based methods are, in general, considered as screening methods.



Optimization and validation assays for ELISA are important aspects of standardizing this technology for GMO detection. Assay validation for food analysis is complex, considering the large diversity of food matrices.

Factors affecting optimization include: (1) selection of parameters (e.g., quality of kits, methods to test modified proteins, and incubation times); (2) selection of thresholds (e.g., limits for positive and negative tests); (3) tracking of controls; and (4) the experience of the laboratory in performing tests.

Factors affecting validation include: (1) extraction efficiency, (2) accuracy of results, (3) precision and ability to distinguish between closely related values, (4) sensitivity limit of detection, (5) specificity, (6) reproducibility, and (7) consistency of detection.

Given the complexity of food matrices, a practical approach to validate results is to use standard reference materials with known concentrations of GMOs in a matrix similar to that of the test sample.

The ELISA is the method of choice to screen for a particular GMO in raw material, semiprocessed foods, and processed ingredients, provided that the expressed protein is not degraded and can be detected. However, because ELISA has lower detection power than PCR methods, it is less sensitive for testing finished food products with many ingredients, especially if the threshold for detection is low.

In general, the theoretical detection limit of an ELISA lies at nanogram protein per mL or at parts per billion. The detection limit for GM soy is 0.01% in 100% soybean flour (Stave et al., 2004). For DNA-analytical approaches, a detection limit of at least 0.01% is described (JanKiewicz et al., 1999).

Important Limitations of Protein-Based Methods

- Protein-based methods can only be applied if a new protein is expressed in the GMO. Using the antisense or sense technology to suppress or overexpress the transcription/translation of a gene originated from plants does not result in an additional protein.
- Newly expressed proteins often vary in expression levels in different plant tissues. The new protein derived from the genetic modification will thus be expressed (e.g., in certain corn varieties) at only very low levels, thus being below the detection limit of the method.
- Proteins like CP4-EPSPS appear naturally among all plant species. It is important to set the level of sensitivity of methods so that GM-derived material is clearly differentiated from conventional crops.

DNA-BASED DETECTION METHODS

DNA-based methods for the detection of genetic modifications consist of (a) the extraction and purification of amplifiable nucleic acids, (b) the amplification of a part of the inherent material specific for the genetic modification by PCR, and (c) the confirmation of the amplified PCR product.



In samples where the GM DNA is present, all exogenous DNA can, in principle, be suitable for GMO detection: promoter sequences, the introduced gene itself, endogenous terminator sequences; and the marker genes used for selection of transformed organisms.

In practice, the choice of primer for PCR testing must be made with great care. As both the 35S promoter of CaMV and the NOS terminator of *Agrobacterium tumefaciens* occur naturally, the presence of one of these sequences is not necessarily evidence for the presence of transgenic material. However, the presence of both sequences in a sample indicates the presence of GM material. The optimal strategy to test for sequences that do not occur naturally is to amplify overlapping areas comprising the promoter and gene, i.e., a sequence arrangement that has been created in a laboratory. This has been done successfully with glyphosate-resistant crops (Roundup Ready cotton and soybeans) and the insect resistant maize (Bt maize).

Nucleic Acid Extraction

In general, 100–200 mg of the sample are sufficient to yield enough DNA for the subsequent PCR step. To check for potential contamination during the extraction process, an extraction control where buffer or water replaces the sample must be used. In the subsequent PCR, this extraction control must generate a negative result.

The nucleic acid (DNA) concentration may be determined photometrically, fluorometrically, or by gel electrophoresis followed by ethidium bromide staining. Obtaining a suitable quality and quantity of genomic DNA from the sample to be investigated is the first and most critical step for successful PCR analysis. Quality of extracted nucleic acid depends on the sample as well as the method used to isolate the nucleic acid. Low concentrations of plant metabolites, e.g., polysaccharides, can cause inhibition of the polymerase used in PCR and result in a false-negative outcome. To identify these inhibitors, a control PCR has to be carried out using primers amplifying a DNA sequence specific for the species from which the sample is derived (e.g., if a maize sample has to be investigated, a maize-specific PCR could be used as an amplification control). If the control PCR is negative, the extracted nucleic acid has to be purified again (Holden et al., 2003).

DNA Amplification

The PCR is an effective method to generate millions of identical copies of a single DNA sequence within a few minutes or hours. Therefore, the adventitious presence of genetic modification can be detected by PCR even if this presence is at extremely low levels (Meyer, 1999). Each cycle in the PCR doubles the number of DNA molecules in the reaction vessel. The reaction components are the limiting factor for this exponential amplification.

Due to the high sensitivity of PCR, contamination with target DNA can lead to false-positive results. Therefore, special care has to be taken to minimize risks for



contamination by carrying out the three steps of PCR in separate rooms and using different equipment in each laboratory.

Primers

The PCR design must be tested using a primer design software program to avoid hairpin structures, primer duplexes, and unbalanced primer annealing temperatures. Moreover, the primer sequences should be run against a sequence database to verify that only the target sequence, and no other, displays significant homology to the primer sequence. However, theoretical biocomputing tests must never replace the empirical testing of specificity and sensitivity of a given PCR detection method.

Target Choice

An appropriate target for the primers is essential for the identification of a GMO. In principle, there are three different strategies as starting points for choosing an appropriated target.

Genetic Elements Commonly Used in GMOs

One could focus on PCR amplification of genetic elements that are commonly used in GMOs, such as the 35-S promoter (P35S) of CaMV or the nos-terminator of *Agrobacterium tumefaciens*, which might suggest the presence of GMOs in case of a positive response. However, these PCR methods will not discriminate between the elements occurring naturally in infected plants or their presence in genetic constructs of GMOs. Especially in the case of rapeseed and other Brassica members, a positive result from a P35S screening may well be a false positive since these plants can be infected by the cauliflower mosaic virus. However, by performing a CaMV-specific PCR based on genes normally not present in GMOs, false positives, as a result of virus infected plants, can be detected.

Gene Construct

The gene construct consists of several elements derived from different sources. Therefore, the junction sequences between two adjoining DNA segments could be the target for a specific detection of the genetic construct. It has to be considered that these two joint elements can be introduced into other organisms resulting in a different GMO containing the same genetic construct. Consequently, this is not conclusive when detecting a specific GMO event.



Event Specific

When the GMO is the result of a nonhomologous recombination, the integration site is unique. Therefore, this plant-construct junction fragment, when it is used as a target for detection, will clearly detect a specific transformation event. Whenever the same gene construct is used to produce different GMOs, this will be the only strategy to distinguish between GMOs containing the same gene construct.

However, a prerequisite for the development of such methods is the sequence information of the GMO as well as the availability of suitable reference material.

Authorized GMOs

The notifier must provide the description of detection and identification techniques for the GMO plant. In addition, information must be provided on genetic modification for the purpose on developing one or several registers, which can be used for the detection and identification of particular GMO products to facilitate postmarketing control and inspection. This information should include, where appropriate, the lodging of samples of the GMO or of its genetic material with the competent authority and details of nucleotide sequence and other types of information necessary to identify the GMO product.

Nonauthorized GMOs

There are actually two situations in nonauthorized forms:

- (a) Sequence data and reference material available.

Event-specific detection systems for these GMOs could be included within both the qualitative and quantitative tests.

- (b) No sequence data and reference material are available.

Indirect Method

In this case, a subtractive approach could be implemented. Samples are tested for elements commonly used in GMOs (screening) and for the presence of authorized and known nonauthorized GMOs with event-specific tests. In case the event-specific tests are negative and a positive test is found with one of the commonly used elements (screening), this could be considered as an indirect evidence for the presence of unknown GMO.



Direct Method

Another alternative to detect nonauthorized GMOs is the fingerprinting method, which consists of amplifying the DNA of seed lots by using random primers (e.g., AFLP) in combination with a GMO screening primer (e.g., P35S, NOS). The fingerprints generated then may be able to differentiate nonauthorized GMOs from authorized ones. This strategy can be considered as a direct way for the detection of nonauthorized GMOs.

Qualitative Analysis

For all three strategies described previously (target choice), qualitative PCR-based detection methods have been developed for maize and soybean (Meyer, 1999).

A general guideline to detecting GMOs in conventional seed lots is to start with a screening method, where different GMOs can be screened in just one assay. However, some GMOs currently on the market do not contain a CaMV35S promoter and/or NOS terminator. Therefore, when using this approach, not all GMOs will give a positive result in PCR. If a positive result for one or more of the genetic elements of a genetic construct has been obtained in PCR, specific methods for a genetic construct and/or a GMO event have to be carried out. If different GMOs, containing the same genetic construct are on the market, an event-specific method is mandatory. Additionally, an isogenic wild type or an equivalent nonmodified line should be used as a control.

In a standard PCR test, factors such as excessive heat, nuclease activity, and low pH (quite common in food processing) contribute to DNA degradation. Compounds present in foods (i.e., proteins, fats, polysaccharides, polyphenols, cocoa extracts, and caramelized sugar) can inhibit DNA polymerase, and data suggest that the critical minimum average DNA size for successful PCR analysis is ~400 bp (Meyer, 1999).

Different methods can be used to confirm the PCR results: (1) specific cleavage of the amplified product by restriction endonuclease digestion (Vollenhofer et al., 1999); (2) hybridization with a DNA probe specific for the target sequence (Council Regulation (EC), 1998; Hill et al., 1999) direct sequencing of the PCR product (Matsuoka et al., 2002).

A collaborative validation study involving 29 laboratories in 13 countries employing qualitative PCR to determine the 35S promoter and the NOS terminator from GM soybeans, maize, and other processed components showed that samples of soybeans and maize containing 2% GMOs were unequivocally identified by all laboratories and correct classification was achieved by analyzing the CaMV 35S promoter in samples containing 0.5% GM soybeans. The method for the detection of the NOS terminator was less sensitive, giving three false-negative results (out of 105 samples analyzed). Because of the large size of the maize genome, this qualitative PCR method was somewhat less sensitive for the detection of transgenic maize for samples containing 0.5% GMOs (Lipp et al., 1999).



Quantitative Analysis

A crucial aspect of analysis of GMOs in food is quantitation because maximum limits of GMOs in foods are the basis for labeling in the EU. Therefore, more quantitative PCR approaches are needed. The PCR was shown to be quantitative if an internal DNA standard was coamplified with target DNA (Hübner et al., 1999a; 1999b; Raeymackers, 1993).

Competitive PCR

The competitive PCR method was popular early in research for food methods, but is rarely used today. It is based on the simultaneous amplification in PCR of the target and a so-called competitor in defined quantities. By comparing the amplification products between the target and the competitor, the amount of target DNA can be estimated. A prerequisite for the analysis is the development of a competitor for each GMO. This is time consuming and is only possible for laboratories with experience in molecular biology. The advantage of the methodology is that no specific equipment is necessary to carry out the investigation.

In systems such as the quantitative-competitive (QC)-PCR method, the presence of PCR inhibitors will be noticed immediately because the amplification of both internal standard and target DNA will be simultaneously affected. The QC-PCR method consists of four steps: (1) coamplification of standard and target DNA in the same reaction tube; (2) separation of the products by an appropriate method, such as agarose gel electrophoresis and staining the gel with ethidium bromide; (3) analysis of the gel densitometrically; and (4) estimation of the relative amounts of target and standard DNA by regression analysis. The amplicon produced from the internal standard is 20–25 bp smaller than that produced from the specific target, making differentiation possible by electrophoresis. The internal standard DNA acts as a competitor to the target sequence during PCR, and equivalence is reached when both are initially present at the same concentration, producing the same amount of each amplicon. This form of PCR involves the effective titration of a set amount of internal standard with different amounts of target DNA. The relative amounts of each amplicon are usually assessed by amplicon intensity, either by staining followed by densitometry or by image-processing software (Hübner et al., 1999b; Raeymackers, 1993).

Single-Competitive PCR

In this case, single pair of amplicons is produced (i.e., to the specific target and its competitor). This method should only be considered semiquantitative, as any degradation of the DNA that may have occurred in the sample is not taken into account in the analysis. It is, therefore, more applicable to raw materials and unprocessed food.

Using a calibrated internal standard (Hübner et al., 1999b) helps make the method more robust and decreases the occurrence of false negatives as a result of



PCR inhibition. Hübner et al. (2001) demonstrated that if the appropriate calibration of internal standard DNA is performed, a good correlation ($r^2 = 0.995$) can be obtained and that such a calibration is a prerequisite for quantitation. Threshold analyses can be performed by making the point of equivalence between the two amplicons represent a GMO content of 1%; this is achieved by adjusting the amount of internal standard added to each reaction.

Double-Competitive PCR

This technique is a two-stage process, which takes into account both the fragmentation and amplifiability of the DNA. Its use in detecting transgenic material in foods has been described elsewhere (Wurtz et al., 1999). An initial examination is made of the amount of amplifiable species-specific DNA present in the sample. Soya content is usually assessed by using the Lectin Le 1 gene and in maize by using the Invertase Ivr gene. The sample DNA extract to be analyzed is then diluted accordingly to match the concentration of the Soya lectin or maize invertase competitor. A second set of competitive PCR reactions is then performed to specifically detect the modification in question. It is usual to adjust the internal standard DNA concentration appropriately to an equivalent of 1% GMO content.

These two methods are not relevant today and the most commonly used method for GMOs food analysis is real-time PCR.

Real-Time PCR

Real-time PCR measures the amount of molecules produced during each cycle of PCR rather than just at the end. It employs fluorescent probes that anneal to the DNA to measure the amount of target molecules amplified. There is a variety of real-time PCR methods, with a range of different chemistries and instrumentation. The aim, however, remains the same: to follow the PCR reaction and the production of specific amplicons cycle by cycle. This requirement negates the major drawback of gel-based systems, which is to produce sufficient amplicon for visualization by staining an agarose gel.

Since the denaturation of DNA during food processing varies significantly from process to process, neither of these PCR techniques is directly suitable for determination of the absolute amount of transgenic material used at the start of the food processing. This is, however, required by the EU regulations, which are based on the level of ingredients.

The amount of transgenic ingredients in the food can be extrapolated by using genes of similar copy number as the internal standard, under the assumption that all DNA will be denatured to the same extent during the food processing.

Instead of quantifying just the presence of the transgenic sequence, an additional test is performed to quantify a reference gene known to be present in the ingredient at 100%. Although the DNA will be degraded during the processing, the relative amounts of reference sequence to transgenic sequence will remain the same (Ahmed, 2002).



In theory, production of PCR products should proceed exponentially. However, in practice it reaches a plateau between 30 and 40 cycles because certain reaction components become limiting (Mangana-Vougiouka et al., 1999).

It has been shown that the concentration of DNA in real-time PCR reaction is proportional to PCR cycle number during the exponential phase of PCR. Therefore, if the number of cycles it takes for a sample to reach the same point in its exponential growth curve is known, its precise initial DNA (then GMO) content can be determined. Several commercially available real-time PCR thermal cyclers automate the analytical procedure and allow cycle-by-cycle monitoring of reaction kinetics, permitting calculation of the target sequence concentration. Several formats are used to estimate the amount of PCR product: (1) the ds-DNA-binding dye SYBR Green I; (2) hybridization probes or fluorescence resonance energy transfer (FRET) probes; (3) and hydrolysis probes (TaqMan technology).

The ds-DNA-Binding Dye SYBR Green I

The SYBR Green I dye binds to the minor groove of the DNA double helix. The unbound dye produces little fluorescence by itself, but this is enhanced significantly when it binds to DNA. The resulting fluorescence is measured at the end of the primer elongation stage of PCR. This method lacks amplicon specificity and, hence, can be used in all assays of this type. It has the added advantage that a melting curve analysis can be performed after PCR, which gives added confidence to the result because nonspecific products tend to melt at a much lower temperature than do the longer specific products (Wiseman, 2002).

Hybridization Probes

This format is used whenever maximum specificity is required with regard to amplicon identification. Two sequence-specific oligonucleotides labeled with fluorescent dyes are included in the PCR reaction, the first has a fluorescein label and the second an LC Red 640 label. The design of these oligonucleotides is crucial to optimal performance because both must hybridize within one to five nucleotides of each other on the DNA template. If no hybridization to the target DNA takes place, the fluorescein-labeled oligonucleotide, once excited, simply emits green fluorescence at longer wavelengths. However, after hybridization and amplicon production, both labeled oligonucleotides hybridize in close proximity to each other. The emitted energy from the fluorescein-labeled oligonucleotide is transferred by fluorescence resonance energy transfer (FRET) to the adjacent LC Red 640-labeled oligonucleotide, which then emits red light at a longer wavelength. This red light is measured and used to follow the PCR in real-time and is proportional to amplicon generation (Wiseman, 2002).



Hydrolysis Probes

The Taqman™ chemistry exploits the 5'-3' exonuclease activity of particular *Taq* polymerases, which produce a fluorescence signal as a result of the hydrolysis of a fluorescently labeled template-specific oligonucleotide included in the PCR (Heid et al., 1996). The signal generated is proportional to the amount of specific amplicon produced during the PCR. It is detected by a sensitive charge-coupled device, which allows the earliest possible differentiation between the baseline and the true signal. At this early stage, the PCR is operating in an exponential manner that has not yet been subjected to inhibitory kinetics. The point at which the fluorescence signal crosses a predetermined value is recorded and referred to as the cycle threshold (Ct). As with competitive PCR, two-PCR systems are used to obtain a quantitative result: one to detect the specific transgene and the other to detect a plant-specific DNA sequence that can be used for relative quantitation. These are followed by using different fluorescent reporters. Subsequent analysis generates two standard curves and allows the initial ratio of the templates in the reaction to be determined (Vaitingom et al., 1999). The small amplicons (about 80 bp) generally used facilitate better kinetics during the PCR and are equally applicable to unprocessed or processed foods and complex food matrices and allow the relative ratios of individual transgenes to be determined. Occasionally, the fluorescently labeled probe can be subjected to hydrolysis in some food matrix systems (Wiseman, 2002).

VALIDATION

Method validation is the process of showing that the combined procedures of sample extraction, preparation, and analysis will yield accurate, precise, and reproducible results for a given analyte in a specified matrix. In order to validate an analytical method, the testing objective must be defined and performance characteristics must be demonstrated. Performance characteristics include accuracy, extraction efficiency, precision, reproducibility, sensitivity, specificity, and robustness. The use of validated methods is important to assure acceptance of results produced by analytical laboratories. According to European Union legislation, state laboratories participating in public food control should, whenever possible, use analytical methods that have been tested. This is also the case for all laboratories aiming at accreditation.

After initial "in house" validation, ideally, each new method should be performed in numerous laboratories to demonstrate reproducible, sensitive and specific results. In a collaborative assessment experiment, the same measurement should be assessed on identical material (Lipp et al., 1999; Stave et al., 2000).

Validation Parameters for Quantitative PCR Methods

It is generally assumed that the specific transgene and plant-specific templates degrade equally in all instances and that the amplicons produced during PCR are



about the same size. One of the most important criteria in these analyses is that sufficient DNA is added to each reaction to ensure that an appropriate number of copy-specific endogenous targets are present. If this cannot be achieved, quantitative problems will arise. In the following sections, the availability of DNA, free of PCR inhibitors, and in sufficient quality and quantity are assumed.

Specificity and Selectivity

Specificity is defined as the ability of a method to detect a substance or a class of substances without impairment by other components present in the sample and to identify the analyte unambiguously. Selectivity is the ability of a method to detect different components in parallel without reciprocal interference and to identify the analyte unambiguously. Quantitative PCR detection methods for GMO food analysis must be highly specific. If multiplex quantitative PCR detection methods are applied, they must also be selective (Markoulatos et al., 2001, 2002).

Specific detection systems for plant species like wheat are difficult to achieve because of the presence of homologous DNA sequences in closely related plant species. The specificity of a detection system must be tested with DNA from closely related agricultural plants in addition to the most important plants in food production, such as wheat, rice, corn, potatoes, soybeans, rye, barley, oats, and millet.

Like all cereals, corn (*Zea mays*) belongs to the family of *Gramineae*. Thus, the most important agricultural plants of the family *Gramineae* must be tested, and no target amplification should be detectable using plant genomic DNA originating from wheat (*Triticum aestivum*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), oats (*Avena sativa*), soy (*Glycine max*), and rye (*Secale cereale*). Several varieties of *Zea mays* should be tested to verify that the copy number of the target sequence does not vary within the plant species of interest. This aspect is important for GMO testing because the analytically determined amount of corn DNA will be used to calculate the GMO content of the corn-derived ingredient. The gene coding for zein, a storage protein in *Zea mays*, was described for detecting the presence of maize DNA. A zein gene-specific QC-PCR was developed by constructing the corresponding competitor. Upon specificity testing of this QC-PCR system, it was found that the measured ratio of the band intensities between the target band and the competitor band ranged from 4.6 (Bt11 corn) to 0.27 (BtMON810 corn), suggesting that the copy number of the zein gene can vary up to 15-fold among different maize varieties (Hübner et al., 2001). Because this detection method would lead to incorrectly calculated GMO contents of corn-derived ingredients, the system was not suited for routine analysis and was abandoned. Likewise, many cultivated crop plants contain a high number of gene duplications as a result of the breeding processes.

Sensitivity (Limits of Quantitation and Detection)

The sensitivity of quantitative PCR detection methods can be expressed in terms of limits of quantitation (LOQ) and limits of detection (LOD). These two



validation parameters depend on the amount of genomic DNA used for PCR, in the genome size of the investigated plant species, and on the number of transgenes per genome.

A 200 ng sample of maize DNA contains about 40,000 genome copies. Thus, a GMO content of 0.1% corresponds to 40 copies of the transgenic genome. The minimal number of copies that still can be quantitated depends on the accepted analytical error and on the sensitivity of the PCR detection method. Based on the assumption that the 200 ng plant-template DNA is used for PCR, the theoretical limits of quantitation were calculated and compiled. For GMO food analysis, a minimal LOQ around 0.1% GMO is recommended. Although the LOD of optimized PCR methods is in the range of 1–10 copies of the target sequence, no quantitation should be attempted below the LOQ. Applying soybean and Roundup Ready soybean-specific real-time PCR methods, the LOQ and LOD were experimentally determined and found to be close to the 35 copies (Ankilam et al., 2002; Hübner et al., 2001).

Accuracy

The accuracy of an analytical method is determined by precision and trueness. Both validation parameters are experimentally determined by repeated measurements. For determination of repeatability, these measurements must be made within the same day by the same operator using the same thermocycler. The precision of the tested methods is usually expressed in terms of the relative standard deviation (RSD), which is the ratio of the standard deviation of the mean divided by the mean value of the measurements.

For determination of the precision of QC-PCR detection systems, analysis of at least five replicates must be performed at three different concentration levels (at the equivalence point, one order of magnitude below the equivalence point, and one order of magnitude above the equivalence point). For determination of the precision of real-time PCR detection systems, at least five replicates should also be measured at three to four concentrations, separated by one order of magnitude (e.g., 0.1, 1, 10, and 100% GMO). The RSDs for QC-PCR and real-time PCR detection methods were determined to range from 5% to 20% (Hübner et al., 2001).

Range of Quantitation

The range of analyte concentrations that leads to acceptable results determines the range of quantitation of a given method. The QC-PCR method can be used for quantitation in the range of two orders of magnitude: from one order of magnitude below the equivalence point to one order above the equivalence point. Changing the equivalence point by using different amounts of competitor DNA requires the experimental reassessment of the range of quantitation. Using real-time PCR, the lower limit of the working range corresponds to the LOQ described previously. The upper limit of the range of quantitation corresponds to a GMO content of 100%, provided that the amount of genomic DNA used for real-time PCR is not



inhibitory. A maize-specific real-time PCR detection method can be used for quantitation in the range of three orders of magnitude, whereas for plants with smaller genome sizes, such as soybeans, the range of quantitation for real-time PCR expands to four orders of magnitude (Hübner et al., 2001).

ACCREDITATION

A laboratory carrying out GMO analyses should participate in an internationally recognized external quality control assessment and accreditation scheme. Council Directive 93/99/EEC (Hübner et al., 2001) and Article 7 of Council Directive 89/397/EEC oblige EU member states to take all measures necessary to ensure that laboratories comply with the general criteria for the operation of testing laboratories, which are laid down in European Standard EN 450001 and supplemented by standard operating procedures.

PRESENT AND FUTURE OF GMO DETECTION

Currently most laboratories analyze products for the qualitative presence of GM material by straightforward PCR. The imposition of threshold levels by various regulatory bodies makes it likely that quantitative versions of PCR increasingly will become the focus of attention.

It can be anticipated that the situation concerning the detection of GMOs will become more complicated in the near future. Many other GM varieties, besides the few that have now received market approval in the EU, are in the pipeline for approval here or are already on the market in important trading partners of the EU. For the maintenance of European regulatory requirements it will, therefore, be necessary to develop or implement more powerful screening and detection methods in the near future. The most prominent example of an innovative and promising approach is the application of the microarray technology for GMO detection. When testing, for instance, for the presence of GMOs, the microarray, in principle, can detect, identify, and quantify large numbers of GMO varieties present in a sample in one single assay. At this moment, due to the fact that the current available linear labeling of the DNA does not give the required increase in fluorescence signal, the microarray technology is still used as a first-line screening approach. A second step will then be necessary to more exactly quantify the presence of detected GM varieties. The DNA microarray technology is very flexible since new varieties can be included in the screening procedure by adding additional sequences to the array.

One possible problem in the application of such chips to food testing is that with matrices as complex as food, the systems may not yet be robust enough to deliver reliable and reproducible results.



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