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Review Paper

Application of DNA and Immunoassay Analytical Methods for GMO Testing in Agricultural Crops and Plant-Derived Products

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Abstract: The introduction of new agricultural commodities and products derived from modern biotechnology may have an impact on human and animal health, the environment and economies of countries. As more Genetically Modified Organisms (GMO) enter markets worldwide, the monitoring of GMOs is being preferred for obvious reasons such as determination of seed purity, verification of non-GMO status of agricultural crops and fulfilling GMO labeling provisions, to mention a few. Numerous GMO analytical methods which include screening, identification and quantification have been developed to reliably determine the presence and/or amount of GMO in agricultural commodities, in raw agricultural materials and in processed and refined ingredients. The detection of GMOs relies on the detection of transgenic DNA or protein material. For routine analysis, a good sample preparation technique should reproducibly generate DNA/protein of sufficient quality, purity and yield while minimizing the effects of inhibition and contamination. The key sample preparation steps include homogenization, pretreatment, extraction and purification. Due to the fact that analytical laboratories receive samples that are often processed and refined, the quality and quantity of transgenic target analyte (e.g. protein and DNA) frequently challenge the sensitivity of any detection method. With the development of GMO analysis techniques, the Polymerase Chain Reaction (PCR) technique has been the mainstay for GMO detection, and the real-time PCR is the most effective and important method for GMO quantification. The choice of target sequence; for example a promoter, a terminator, a gene, or a junction between two of these elements, is the single most important factor controlling the specificity of the PCR method. Recent developments include event-specific methods, particularly useful for identification and quantification of GM content. Although PCR technology has obvious limitations, the potentially high degree of sensitivity and specificity explains why PCR in its various formats, is currently the leading analytical technology employed in GMO analysis. Comparatively, immunoassays are becoming attractive tools for rapid field monitoring for the integrity of agricultural commodities in identity preservation systems, whereby non-specialised personnel can employ them in cost-effective manner. This review discusses various popular extraction methodologies and summarises the current status of the most widely used and easily applicable GMO analysis technologies in laboratories, namely the PCR and immunoassay technologies.

Keywords: Genetically modified organism, polymerase chain reaction, real-time polymerase chain reaction, enzyme-linked immunosorbent analysis, reference materials

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INTRODUCTION

Background

As soon as human beings settled, they started to grow plants and breed animals. Gradually, they artificially created new species by selecting some types of plants and animals. However, up to a few years ago, human beings were unable to directly control the biological process of this creation which is based on a random mix of genetic information through sexual reproduction. Though human beings have improved their techniques to control sexual reproduction, they have not yet acted directly at the core of the biological mechanism. They did not modify the genetic information that living organisms harbour.

Life sciences have undergone enormous progress since the discovery that molecules bear genetic information. These molecules are called nucleic acids, among which DNA (deoxyribonucleic acid) plays a central role. The knowledge of the structure and the biochemistry of DNA and the mechanism of DNA synthesis (polymerization, ligation, and cutting) have allowed biologists to act directly on DNA sequences.

Researchers have taken advantage of this manipulation of DNA and of the genetic code to associate DNA sequences coming from different organisms. They have succeeded in building new DNA molecules by recombining different DNA sequences with molecular biology techniques. DNA is like a magnetic tape–it can be cut, moved and reinserted to create new or different information. With the advent of molecular biology, researchers have succeeded in integrating foreign DNA, not only within plant genome DNA, but also in mouse or rat DNA.

What is Genetically Modified Organism?

Genetically Modified Organisms (GMOs) are living organisms whose genomes have been modified as a result of gene technology, resulting in the introduction, removal or alteration of a specific characteristic or 'trait'. Gene technology enables the exchange of genes not only within species but also across boundaries, and between organisms that are not sexually compatible. The novel DNA 'construct' contains all the information needed to produce the new 'trait' which also includes the production of a novel protein.

The first genetically modified (GM) fruit sold on the market was the well-known FlavrSavr tomato, which was designed to soften more slowly. A slower softening process means that the tomato can stay on the vine for longer which makes them tastier. Furthermore, tomatoes with elevated levels of antioxidant lycopene may protect against cancer. Currently, the most widely inserted genes in GMOs confer resistance to worms, insects or to herbicide.

Benefits of GM Technology

Malnutrition plays a significant role in half of the nearly 12 million deaths each year of children under five in developing countries. In addition due to lack of food, deficiencies in micro-nutrients (especially vitamin A, iodine and iron) are widespread (Unicef, 1998).

Changes in the patterns of global climate and alterations in use of land will exacerbate the problems of regional productions and demands of food. Dramatic advances are required in food production, distribution and access if these needs are going to be addressed. Some of these advances will occur from non-GM technologies, but others will come from advantages offered by GM technologies (Royal Societies Report, 2000).

The development of GMOs offers potential of increased agricultural productivity or improved nutritional values that can contribute directly to enhancing human health and development. From a health perspective, there may also be indirect benefits such as reduction in agricultural chemical usage, enhanced farm income, crop sustainability and food security, particularly in developing countries (World Health Organization Report, 2005).

Table 1: Expressed traits and associated genes that have been incorporated into animal feed crops

Trait	Genetic elements (s)
Insect resistance	Cry1Ab; Cry1Ac; Cry9C; Cry3C; Cry1F
Glufosinate herbicide resistance	Phosphinothricin N acetyltransferase
Glyphosate herbicide tolerance	5-enolpyruvylshikinate-3-phosphate synthase (EPSPS)
Male sterility	Barmase ribonuclease
Sulphonyl urea herbicide tolerance	Variant form of acetolactate synthase
Oxynil herbicide tolerance	Nitrilase
Modified seed fatty acid profile	Delta-12 desturase
Virus resistence	Coat protein; helicase/replicase

(Adapted from MacKenzie *et al.*, 2002)

Genetically Modified Crops Used in Animal Feed

As the global land area of biotechnology-derived crops modified for agronomic input traits such as herbicide tolerance and/or insect resistance continues to increase, these crops have become an increasingly important source of feedstuffs for farm animals.

As a source of livestock feed components, the relevant GM crop species include canola (rapeseed), maize (corn), soybean, cottonseed and potato. These species have been modified to express, either singly or in combination, the traits of insect resistance, herbicide tolerance, or in the case of potatoes, resistance to virus infection (Table 1). Many of the proteins that have been expressed in GM plants in order to confer these traits are already present in plant products or in other agricultural products (MacKenzie *et al.*, 2002).

Animal products such as meat, milk and eggs are significant sources of high-quality food for humans and represent approximately one-sixth of their food energy and one-third of their food protein on a global basis (Council for Agricultural Science and Technology, 1999). Diets for farm animals may contain forages (e.g. pasture, hay and silage), crop residue (e.g. maize stover and rice straw), cereal grains, and food and fiber co-products (e.g. soybean, canola and cottonseeds meals, cottonseed hulls and corn distillers' dried grains).

A study was carried out in Poland (Sieradzki *et al.*, 2006) to detect, identify and quantify GMO by analyzing DNA in animal feed. Fifty nine of the 109 examined feed samples contained GM crops. The presence of GMO was detected in 31 samples of soybeans and soya meal, 4 samples of maize and 24 samples of feed mixtures. The most commonly used GMO in animal feed in Poland was Roundup Ready soya, found in 57 samples. GM maize was found in only 2 of the 69 samples of maize and compound feed. In addition, 2 maize samples were contaminated with Roundup Ready soya.

In Malaysia, two small studies also demonstrated presence of GMOs in animal feed. In the first study carried out in 2004 (Nguyen, 2004) on 23 animal feed samples that were purchased commercially, 11 were tested positive for Roundup Ready soy. The second study in 2002-2006 (Cheah, 2006), tested for both the EPSPS and Cry1A(b) amplification in 13 animal feed samples that were purchased commercially. 12 samples were tested positive for EPSPS gene whereas 11 were tested positive for Cry1A(b).

Global Status of Commercialized GM Crops

Listed below are some noteworthy figures (James, 2006) regarding cultivation of GM crops world wide:

a) In 2006, 22 countries grew biotech crops; 11 developing countries and 11 industrial countries. They were, in order of hectarage: USA, Argentina, Brazil,

Canada, India, China, Paraguay, South Africa, Uruguay, Philippines, Australia, Romania, Mexico, Spain, Colombia, France, Iran, Honduras, Czech Republic, Portugal, Germany and Slovakia.

b) In 2006, the USA followed by Argentina, Brazil, Canada, India and China were the six principal adopters of GM crops globally, with India for the first time replacing China at number five in the world ranking by planting more Bt cotton than China.

c) Spain continued to be the lead country in the European Union (EU), planting approximately 60,000 hectares in 2006. Slovakia, a new EU member, joined the other five EU biotech crop countries (Spain, France, Portugal, Germany, Czech Republic) in planting biotech crops.

d) In 2006, the global biotech crop area continued to soar, when for the first time 10.3 million farmers in 22 countries planted 102 million hectares of biotech crops, compared to 90 million hectares planted by 8.5 million farmers in 2005.

e) Over the last eleven years, 1996 to 2006, the global biotech crop area increased more than sixty-fold in the first eleven years of commercialization, making biotech crops the fastest adopted crop technology in recent history.

f) Soybean continued to be the principal GM crop in 2006, occupying 57% of global biotech crops area, followed by maize (25%), cotton (13%) and canola (5%).

g) In 2006, herbicide tolerance in soybean, maize, canola, cotton and alfalfa continued to be the most dominant trait (at 68%), followed by Bt insect resistance (19%) and stacked traits at 13%. Stacked traits were the fastest growing trait groups between 2005 and 2006 with 30% growth, compared with 17% for insect resistance and 10% for herbicide tolerance.

h) Global accumulated impact of GM crops for the decade 1996 to 2005, in terms of net economic benefits to biotech crop farmers, was 13 and 14 billion US dollars for developing and industrial countries respectively.

Issues of GMO Safety

Conflicting assessments and incomplete substantiation of the benefits, risks and limitations of GM food and feed have added to existing controversies. The use of GMOs may involve potential risks for human health and development because many, but not all genes used in GMOs have been used in the food supply chain before. Therefore while other new types of food crops are usually not subject to safety assessment before marketing, such assessment of GM foods were introduced before the first crops were developed (World Health Organization, 2005).

Whilst Europe has raised many issues regarding GM crops, undoubtedly the major concern relates to the safety of the food and feed derived directly or indirectly from such crops. Of particular concern is the need for reassurance regarding the fate of DNA and resultant proteins derived from introduced traits. This has led to several important questions (Beever *et al.*, 2000):

a) could the DNA of the inserted or modified genes, or their products, if transferred to animals, cause adverse health effects in these animals;

b) could these DNA fragments or proteins be transferred to and accumulate in the products (milk, meat, eggs) of animals fed GM crops; and

c) will consumption of agricultural crop material or animal products derived from GM crops lead to adverse health effects in human.

Safety assessments conducted by regulatory agencies use scientific, risk-based methods to evaluate the novel trait(s) and, ultimately, the new crop. A comparative assessment process identifies similarities as well as intended and unintended differences between novel and conventional crops and their food and feed products. Intended effects are the desired change(s) in the new crop that

are the result of the genetic modification. Unintended effects include all other differences between the new crop and its conventional counterpart and encompass predicted and unexpected changes (Council for Agricultural Science and Technology, 2006).

Regulation of GMOs

By now, many countries, including US and the European Union have introduced legislation regulating the approval and release of GMOs. For example, in the US, three independent authorities are involved in the regulation of the release of genetically engineered plants and their use as foodstuffs: APHIS (Animal and Plant Health Inspection Services), FDA (Food and Drug Administration) and EPA (Environmental Protection Agency). The APHIS controls movement between states, importation and culture assays of GMOs that might induce diseases in plants or are inherently disease organisms. The FDA makes rules for additives and new foodstuffs, except meat, and products coming from poultry farming. The FDA makes rules for animal medicines and is also concerned with the labeling of food. Currently, the administration considers that the composition of a food consisting of or derived from genetically modified plants does not differ significantly from its conventional counterpart. Thus, the labeling of GMOs is not mandatory in the US (Gachet *et al.*, 1998).

By comparison, within the EU, the traceability and labeling of GMOs is regulated by Regulation (EC) No. 1830/2003. This regulation sets out to ensure that relevant information concerning any genetic modification is available at each stage of placement of GMOs on the market and that the food and feed is accurately labeled. Additionally, Regulation (EC) No. 1829/2003 on GM food and feed stipulates that products containing material known to be of GM origin and not having been the subject of an identity-preserved (IP) scheme must be clearly labeled. Materials of non-GM origin produced under an IP scheme are subject to a *de minimis* threshold of 0.9% for adventitious contamination with EU approved GM varieties, and products that are below this limit do not need to be labeled. For non-EU approved varieties, the limit is set at 0% unless the variety has been given a favourable scientific review, in which case it is 0.5%. These thresholds refer to the proportion of GM food in any single ingredient of a foodstuff which are generally determined on a gravimetric basis. Ingredients that bear no information to indicate GM origin, but which have not been produced under the IP system trigger labeling if the smallest amount of GM DNA is detected.

In Malaysia, the Biosafety Bill was approved by Parliament in July 2007 allowing regulations pertaining to labeling of GMOs in food and feed to be introduced and enforced in the near future.

As the GMOs bear supplementary genetic information in comparison with the conventional plants, the purpose of the analytical methods used for enforcement is to target these foreign DNA sequences. The control and detection of such foodstuffs is possible due to the Polymerase Chain Reaction (PCR) analytical method in addition to the conventional protein detection tests using antibodies like Enzyme-linked immuno sorbent assay (ELISA) test.

EXTRACTION METHODS

The crucial influence of extraction technique and sample matrix properties on the results of GMO quantification has been demonstrated in a study (Cankar *et al.*, 2006). Appropriate extraction techniques for each matrix need to be determined to achieve accurate DNA quantification. Due to food processing, the DNA in sample matrixes can be present in very low amounts and also degraded. The extraction method must ensure high yield and quality of the DNA obtained and must be carefully selected, since even components of DNA extraction can influence PCR reactions (Cankar *et al.*, 2006). The aim of the extraction

procedure is to isolate DNA or protein of suitable integrity, purity and quantity to allow subsequent analysis. In order to select the most appropriate DNA or protein extraction procedure, consideration should be given to the sample matrix, the target analyte and the type of analysis required, for example for screening, qualitative or quantitative purposes (Terry *et al.*, 2002). The sample storage history and the degree of processing of the foodstuff will also influence the integrity of the target analyte.

DNA – The Fundamental Building Block of Life

DNA is present in all living organisms, with the exception of some viruses. DNA provides the genetic blueprint or code for an organism and is identical in each cell of a particular organism. Comparatively, the protein composition varies considerably from one cell type to another within the same organism and can also vary at different stages of the cell's life cycle.

DNA contains all the information necessary to synthesize proteins – the molecules that provide the functionality of an organism. However, DNA not only encodes this information but also the instructions to control the amount of each protein synthesized and where and when in the organism a protein will be synthesized. DNA consists of two strands of complementary sequences that are held together by hydrogen bonds. These strands play a pivotal role in the PCR process. The two strands are interwined forming the DNA double helix (Griffiths *et al.*, 2003).

The complete set of DNA sequences present in each cell of a given species is referred to as the species' genome. The DNA amount in the unreplicated, haploid nuclear genome of an organism is referred to as its C-value. The DNA content of the unreplicated haploid complement is known as the 1C value. DNA amounts are usually expressed in picograms (pg) or in megabase pairs of nucleotides (Bennet *et al.*, 2000). Based on the estimated 1C values for various crops, 100 ng of DNA (which is the typical amount used in a PCR reaction) may contain approximately 8 x 10^4 copies of soybean genome or 3.8 x 10^4 copies of maize genome. This corresponds to a total of 80 genome copies for soybean or 38 genome copies for maize if only 0.1% of 100 ng of DNA is of GMO origin. This information proves vital when used in quantitative PCR for plotting calibration curves.

Sampling

One of the major considerations in analytical testing of almost any product is the sampling procedure. The sample analyzed must be representative of the material from which it is taken otherwise the testing regime is flawed.

The sampling procedure determines the how representative is the result, whereas quality and quantity of analytes may vary depending on the sample preparation. The sample plan and sample size have to meet statistical requirements with respect to homogeneity and threshold limit up to which the result should be reliable.

The more heterogeneous the material, the more difficult the sampling procedure as the degree of distribution of ingredients will be less uniform. Whilst raw materials may have a high degree of heterogeneity, a more consistent distribution is normally found for processed foods. However, processed foods may have more than one potential source of GMO and this should be considered in a sampling plan. The lower the threshold limit for GM contamination, which may be contributed by factors such as transportation, sampling equipments, sample dust and laboratory analytical equipments; the greater the demands will be upon the sampling plan.

As an example, a recent EU Commission recommendation (2004/787/EC) provided guidance for sampling and detection of GMOs and material produced from such organisms in the context of Regulation (EC) 1830/2003. One of the principles of this recommendation state that harmonized sampling procedures should be utilized for the purpose of estimating the presence of GMOs and these

procedures should apply to seed and other plant propagating material, food, feed and agricultural lots.

Quantity, Quality and Purity of DNA

As DNA is a rather stable molecule, it is the preferred analyte for almost any kind of sample (raw materials, ingredients, processed foods). Currently, available GMO detection methods operate exclusively at the DNA level and are based on the Polymerase Chain Reaction (PCR). The first and most crucial step for GMO detection is the isolation of the DNA (Gryson *et al.*, 2004). For this reason, there are three key factors that determine the success of DNA detection methods. These are:

a) the DNA quantity - this refers to the amount of DNA extracted.
b) the DNA quality - DNA quality is determined by fragment length and degree of damage. This may be caused by exposure to heat, low pH or enzymatic degradation. Nucleases that cause hydrolysis or depurination could also be a cause. Therefore, DNA quality varies according to the material under examination, the degree of processing to which the sample has been subjected, and the DNA extraction method applied. DNA isolated from processed foods and certain agricultural matrixes is usually of low quality, and available target sequences may be rather short, e.g. 100-400 base pair for soybean protein preparations and processed tomato products (Hemmer, 1997).
c) the DNA purity - DNA purity can be severely affected by various contaminants in food matrices. Contaminants may be substances that originate from the material under examination, e.g. polysaccharides, lipids and polyphenols. Chemicals used during DNA extraction procedure may also act as contaminants, e.g. CTAB (cetyltrimethylammonium bromide or hexadecyltrimethyl ammonium bromide) ROSE and the Alkali method (Zimmermann *et al.*, 1998). Another example is the Taq polymerase (which is the key enzyme used in the PCR reaction) and it is inhibited by polysaccharides, ethylenediamine-tetraacetic acid (EDTA), phenol and sodium dodecylsulfate (SDS).

DNA Extraction Method

Provided that the laboratory sample is representative of the field sample, and that it has been adequately homogenized, aliquots between 100mg and 350mg are adequate for DNA extraction procedures in the laboratory. A vast range of methods is available for DNA isolation, as shown in Table 2 and many of them have been evaluated for their applicability to GMO detection in plant material and plant-derived foods. In general, DNA extraction from plant material has to accomplish the following (Bonfini *et al.*, 2001):

a) breakage of cell walls, which is usually achieved by grinding the tissue in dry ice or liquid nitrogen. In the laboratory, the normal grinding process using a blender is also an acceptable method and widely used.
b) disruption of cell membranes by a detergent (e.g. CTAB [Cethyl-trimethyl-ammonium-bromide] or SDS [sodium dodecylsulfate]) which is, besides EDTA [ethylenediaminetetraacetic acid] and a buffering salt like Tris-HCl, a necessary component of any DNA extraction buffer.
c) inactivation of endogenous nucleases by the addition of detergents and EDTA, a chelator of Mg^{2+}, which is an obligatory co-factor of many enzymes.
d) addition of proteinase K for the inactivation and degradation of proteins, particularly in protocols using DNA-binding silica columns.
e) separation of inhibitory polysaccharides from DNA through differential solubilisation in solutions containing CTAB.
f) separation of hydrophobic cell constituents from DNA, e.g lipids and polyphenols by extraction with an organic solvent like chloroform.

Table 2: DNA isolation methods used for the detection of GMOs in plant material and plant-derived food products

Method	DNA quality	DNA yield	PCR	Material
Wizard method			+	Soybean powder, maize powder
			+	Bruised soy grain, lecithin
			+	Soya leaves
	High	Low	+	Soya products (tofu, flour, lecithin)
			-	Soybean oil
				55 Foodstuffs derived from soybean, corn, rice, sugar beet, tomato, wheat
CTAB method			+	Raw potato
			+	Raw soybean
			+	Raw tomato
	High	Low	+	Soya products (tofu, flour, lecithin)
			+	Foodstuffs derived from potato
CTAB method with QIAquick column			+	Soya products: meal, oil, lecithin, tofu, chocolate, etc.
			-	Soya sauce, refined soya oil
CTAB method with Nucleon Phytopure			+	Maize, potato, soya, sugar-beet, tomato
Nucleon Phytopure method	High	Low	+	Soya products (tofu, flour, lecithin)
Qiagen DNeasy method				
Chelex 100 method	Low	High	+	
Alkali method				
AlkaliX method				
ROSE method				
ROSEX method				
Dellaporta method			+	Maize grains
Hexane/guanidine thiocyanate with gel filtration			+	Raw and purified lecithin
CTAB method with High Pure PCR Template preparation Kit (Boehringer)			+	Bt-maize in silage
Modified QIAamp DAN Stool Mini kit			+	Cacao-derived products
SDS/Rnase method with Magyx silica-magnetite-based solid-phase support			+	Dried soybean, soybean flour, extruded defatted soya acid, alcohol-precipitated soya concentrate, lecithin, maize grits, seasoned corn puffs, salted corn chips

(Adapted from Anklam *et al.*, 2002)

g) separation from the detergent and concentration of DNA by alcohol/salt precipitation. Alternatively, the separation of DNA from other cell components can be achieved via purification on a DNA-binding silica column.

Currently, three different approaches of DNA isolation from plant material and plant-derived products are favoured for GMO detection: the CTAB-method, the Wizard DNA-binding silica column method, or a combination of the two. Even though the yields are low, the quality and purity of the DNA is satisfactorily higher than with other procedures e.g. Alkali method, Chelex 100 method and the ROSE methods (Zimmermann *et al.*, 1998). Magnetic Beads technology has also been successfully developed for automated high-through-put DNA analysis (Hahnen *et al.*, 2002).

The CTAB Extraction Method

The CTAB method is able to extract pure DNA of high molecular weight from plants. The procedure invariably follows the outline above (a to g) but using CTAB as a detergent in the DNA extraction buffer. It appears to be an efficient method for a wide range of plant materials and plant-derived foods and it provides a good separation of DNA from polysaccharides. The CTAB method is actually part of the official protocols for GMO detection according to the German Food Act as well as ISO 21571.

On the other hand, DNA-binding silica columns have proven to be suitable for extraction of good quality DNA, and the use of one of the commercially available kits is described in the official Swiss method for GMO detection.

Extraction of DNA from complex matrixes or food products is not always successful. Failures in extracting detectable DNA levels have so far been reported for items such as soybean sauce and refined soybean oil (Pauli *et al.*, 2000). Before the amplification, DNA must be extracted from samples. It is a purification step in which other types of molecules (proteins, lipids and polysaccharides) are removed. A lot of protocols allow this, but there are two main principles of extraction. Either the crude solution of DNA is cleaned from impurities by different agents, or DNA molecules are fixed on a resin with high DNA affinity before being eluted. The CTAB method is based on the first principle, and the Wizard-extraction method is based on the second principle (Gachet *et al.*, 1998).

Briefly, the CTAB-method is divided into five steps:

a) solubilization of DNA by addition of the CTAB-buffer made with the detergent CTAB (at a final concentration of 20 g/l)
b) denaturation of a large amount of proteins contained in the sample
c) first precipitation of DNA with the second CTAB solution (the concentration of CTAB is lower: 5 g/l)
d) second degradation of residual proteins
e) second precipitation of purified DNA with alcohol addition.

The Wizard Extraction Method

Briefly, the Wizard-method is divided into three steps:

a) solubilization of DNA by addition of the extraction buffer and proteinase K (an enzyme that degrades non-specifically as it cuts the covalent bond that links amino acids)
b) the crude DNA solution goes through a column containing the resin
c) purified DNA is eluted from the resin

The CTAB-method is the basis for the official German method and the Wizard-extraction method has become the official Swiss method (Gachet *et al.*, 1998).

Protein Extraction Method

Food processing influences both GM protein antigen denaturation and extractability from the food matrix, adversely affecting antibody affinity and utility of the antibody-based

protein detection assay. Predominantly, protein tests are limited to the analysis of products in the raw state.

The extraction of protein from the samples involves a lysis step followed by centrifugation. The supernatant containing the extracted protein is then used in the detection step. Lysis conditions can be modified accordingly to the nature of the matrix, for example binding agents or specific buffers can be used. Fatty samples can be treated with hexane before extraction. The most commonly applied methods for analysis of GM protein are commercially available antibody-based GM trait detection kits (Terry *et al.*, 2002).

DNA ANALYSIS METHODS

Classification of Methods for GMO Analysis

There are biological analysis (protein-based methods and DNA-based methods) and chemical analysis tests for GMO testing, as illustrated in Figure 1. While genetic analysis investigates a specific part of the product, such as the isolated nucleic acids or proteins, chemical analysis looks at the product as a whole (Taverniers *et al.*, 2004). Chemical analysis includes phenotypical tests such as bioassays or herbicide sprays, as well as methods that are based on altered fiber structure (near-infrared spectroscopy, NIRS) or chemical composition of GMO product as a whole (by chromatography) (Anklam *et al.*, 2002). Even though several methods have been used, most methods developed for the detection of GMOs focus on detecting DNA and is accepted as reference methods of choice for regulatory compliance testing (Taverniers *et al.*, 2004).

What is the Benefit of Detecting DNA Instead of Proteins?

Nucleic acids are not considered as an interesting class of compounds in food chemistry. Indeed, the nucleic acids present in food have no nutritional value and there is no direct relationship between DNA and food quality. But whereas proteins are thermo-sensitive molecules, nucleic acids are very thermo-stable molecules. Upon processing, food proteins are no longer detectable or are detectable with difficulty because they are degraded. Conversely, nucleic acids are only slightly damaged by heat treatment.

According to Meyer *et al.* (1996), by comparison, the ELISA-test which is based on using antibodies against specific proteins and a technique widely used in pharmaceutical tests, may be around 100 times less sensitive than the PCR method. Furthermore, antibody tests such as ELISA are more laborious and time consuming to develop and to validate than nucleic acid tests. Since the supply of antibodies is derived from laboratory animals, production of antibodies is a slow and difficult task. The dependence on antibody producers is certainly a limiting factor for the protein method. However, with the advent of this powerful and precise tool, a specific nucleic acid can be detected whatever the foodstuff analysed, even in mixtures where the GMO ingredient is present in low concentrations.

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) technology is an exploitation of DNA replication, the mechanism responsible for DNA copying in all living cells and is used to detect a specific DNA sequence. Although the principle was first described as replication repair, it was not until the introduction of thermostable DNA polymerase that it became widely applicable. With two primers, each corresponding to one end of the DNA segment to be amplified by PCR, the DNA segment can be amplified exponentially within reasonable time (usually less than 3 hours to obtain 10^9 copies) (Holst-Jensen *et al.*, 2003).

The PCR allows the amplification of the specific target DNA from as low as one copy to over a million copies. This is achieved by cycling the target DNA, mixed with other specific reagents, through various temperatures. The PCR consists of the target DNA of interest, primers that anneal to the

target DNA, nucleotide triphosphate (dNTP) bases and a thermostable DNA polymerase (Griffiths *et al.*, 2003).

Singleplex PCR is a method that employs one primer pair in an amplification reaction for a sample DNA and can detect only one target DNA sequence in one tube. On the other hand, a multiplex PCR method employs several primer pairs in the same amplification reaction for a sample DNA and can detect multiple target DNA sequences by simultaneous amplification in one tube.

Due to the fact that the number of GM crops is continuously increasing, multiplex PCR would be one useful method for GMO analysis. Several multiplex PCR methods for detection of GMOs have already been published (Hernandez *et al.*, 2005; Huang *et al.*, 2004; Matsuoka *et al.*, 2000). The sensitivity of multiplex PCR however, might be affected when one GMO event is more concentrated than the others, because the amplification from a more concentrated event might inhibit the amplification of another less abundant target.

The major limitations for PCR-based detection of DNA derived from GMOs are access to information about applicable PCR primers and access to DNA suitable for reliable analysis. Although several PCR primer pairs for GMO analysis have been developed and published within the last 5-10 years, many of these primer pairs have a limited range of application (e.g. primers suitable only for screening) (Holst-Jensen *et al.*, 2003). Sequence information describing the genetic modifications is usually kept confidential by biotech companies.

Grinding, heating, acid treatment and other processing rapidly degrade DNA while refining can lead to efficient removal of DNA. As a consequence, many products contain little GMO-derived DNA, and this DNA is often of low quality. Even with access to suitable primers, reliable analytical results may therefore not be achievable due to template DNA restrictions (Holst-Jensen *et al.*, 2003).

The Use of PCR in GMO Testing

In order to use PCR in GMO testing, the analyst must know or predict the exact nucleotide sequences that flank both ends of the target DNA region. Any PCR-based detection strategy will thus depend on the selection of the oligonucleotide primers and the detailed knowledge of the molecular structure and transgenic DNA sequences used in the development of all GMOs.

Besides the well-known points of consideration for the primer selection (e.g. no inverted repeats within one primer, no complementarity of one primer to the other, presence of GC-rich 3'end, etc.), the choice will depend very much on the objective of the GMO analysis (Bonfini *et al.*, 2001). For example, there are at least 8 variants of P-35S used in GMO crops. It should be stressed that the detection of these generic GMO markers indicate that the analysed sample contains DNA from a GM plant, but does not provide information on the specific trait that has been engineered in the plant.

PCR Methods for GMO Screening

The purpose of GMO screening is to gain a firsthand insight into the composition of the food and agricultural products. The objective is to determine if a product contains a GMO or not. The result is a positive or negative statement i.e. whether GMO is present or absent in the product.

Most of the currently approved GMOs worldwide contain any of the three genetic elements that can be targeted for GMO screening. These elements are the Cauliflower Mosaic virus promoter (CaMV 35S promoter), the NOS terminator from the soil bacterium *Agrobacterium tumefaciens* and the kanamycin resistance marker gene (NPTII) (Ahmed, 2002). These sequences also occur naturally in plants and soil micro-organisms, therefore a positive result will not necessary confirm the presence of GMO, but will suggest that it is probable (Anklam *et al.*, 2002). To definitively confirm the presence of a GMO, a sample with a positive signal in 35S and/or NOS screening

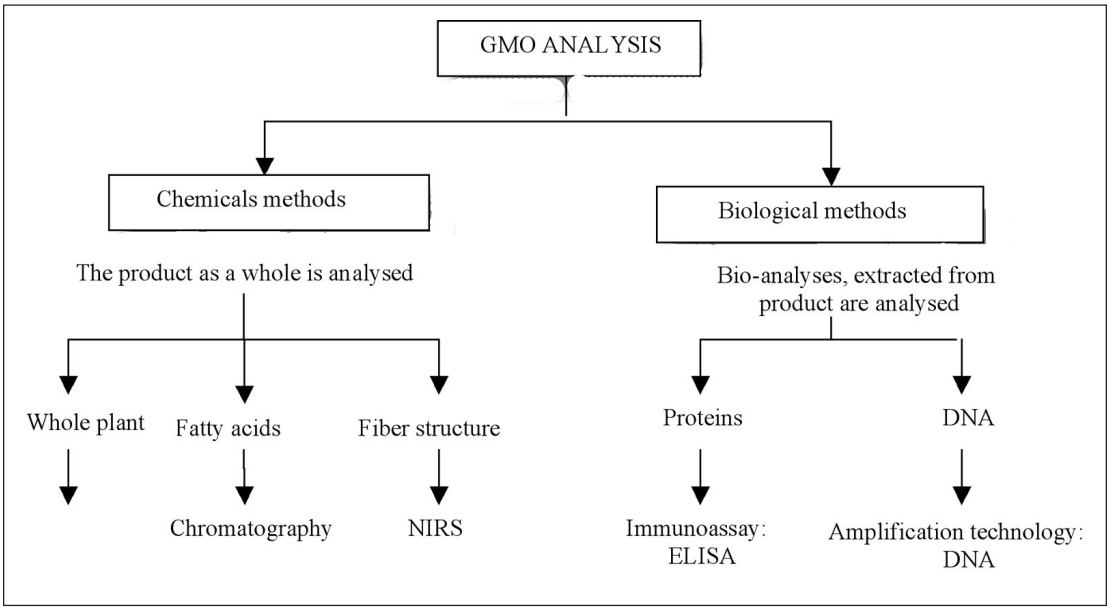

Figure 1: Classification of methods for GMO analysis (Adapted from Taverniers *et al.*, 2004)

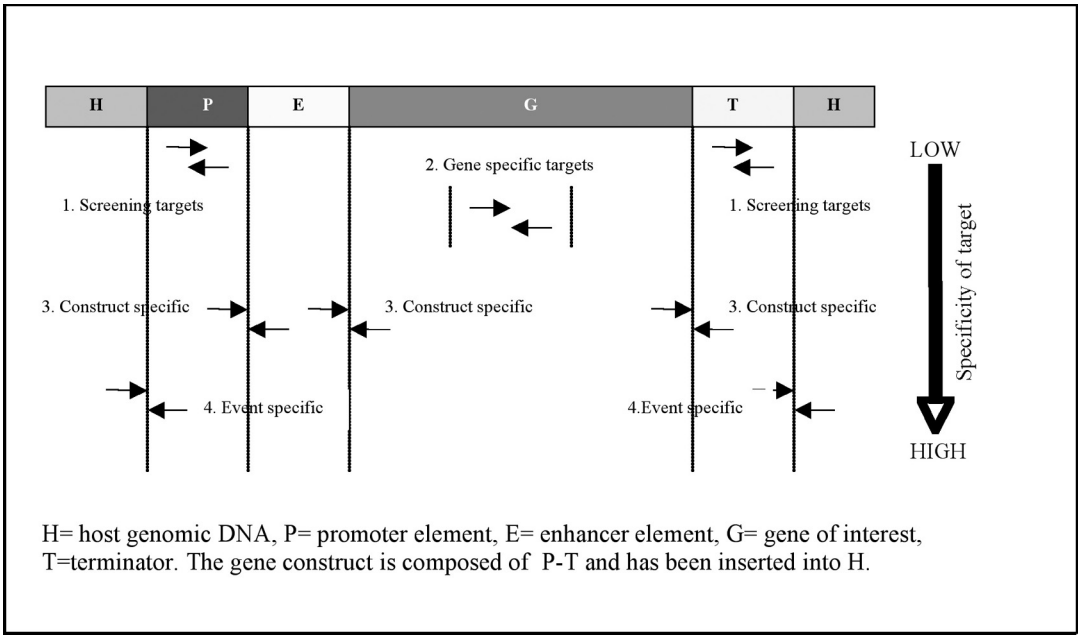

Figure 2: A schematic representation of a typical gene construct and four types of PCR-based assays showing increasing specificity (from top to bottom) (Adapted from Holst-Jensen *et al.*, 2003)

should be further analysed using a construct-specific or event-specific method (Griffiths *et al.*, 2003).

Alternatively, the sample could be analysed for the presence of either naturally-occurring CaMV or *A. tumefaciens* infection respectively. In virus-infected plants or in samples contaminated with plant material carrying the CaMV virus, false positive results can consequently occur. A system for real-time PCR using Taqman groove binder probe has been designed that allows recognition of virus coat protein in virus-infected samples, thus allowing differentiation between transgenic and virus-infected samples. In the study by Cankar *et al.* (2005), the primers did not amplify plant DNA from available GM maize and soybean lines or from different species of *Brassicaceae* or *Solanaceae* that are natural hosts for CaMV.

The NOS terminator sequence is found only in certain strains of *A. tumefaciens,* which are only pathogenic to certain crop species. Moreover, the *A. tumefaciens* frequently found in soil is generally not virulent and does not carry the Ti plasmid, so the NOS gene and its control elements are not present in these naturally occurring strains (Anklam *et al.*, 2002). Comparatively, protein detection methods are less suited to general GM screening, as a single antibody will only recognize one particular protein. There are no structures common to all GM proteins or groups of GM proteins that would allow one antibody to be used to detect a number of GMOs. It is feasible to mix antibodies in order to develop a general screening. However this will not allow quantitation as the binding capacity of each antibody affects the sensitivity of its detection (Griffiths *et al.*, 2003).

PCR Methods for GMO Identification

The purpose of identification is to reveal how many different GMOs are present and if so, if they are authorized with regard to the regulation. This is also referred to as qualitative GMO testing because it helps identify different GMOs in a product. A prerequisite for the identification of GMOs is the availability of detailed information on their molecular make-up. Along with scientific data, molecular registers contain the necessary data for laboratories to design appropriate identification methods essential to fulfill the identification process.

PCR Target Sequences: Categories and Applications

PCR-based GMO tests can be grouped into at least four categories (Table 3) as described by Holst-Jensen *et al.* (2003) corresponding to their level of specificity. Each category corresponds to the composition of the DNA fragment that is amplified in the PCR. For discussion purposes, transformation here means insertion of a gene construct into the recipient organism. The gene construct is composed of several elements, usually at least a gene of interest, a promoter functioning as a start signal, and a terminator functioning as a stop signal for regulation of gene expression as illustrated in Figure 2. In addition, the construct may be flanked by DNA from the cloning vector.

a) Category 1, screening methods

The majority of GM plants have been transformed with constructs containing Cauliflower Mosaic Virus (CaMV) 35S promoter (P-35S) and/or the CaMV35S (T-35S) or

A.tumefaciens nopaline synthase terminator (T-Nos). The most commonly used cloning vectors are pBR322 and its descendants (e.g. pUC19) containing a gene coding for resistance to ampicillin (*bla*) antibiotics, or vectors that contain a gene coding for resistance to neomycin/ kanamycin (*nptII*) antibiotics.

Consequently, PCR methods of category 1 (Table 3) targeting the P-35S, T-35S, T-Nos, *bla* or *nptII*, have wide applications for screening GM material (Agriculture and Biotechnology Strategies Canada Inc GMO database, 2002). However, these screening methods should not be used to

Table 3: Examples of published methods to detect GMOs grouped according to categories of specificity

Type of method	Target sequence
Methods to detect plant-derived DNA	% Chloroplast tRNA gene intron
Methods to detect specific species	% CaMV genomic DNA
	% Maize hmg, invertase and zein genes
	% Rape seed Acetyl carboxylase coenzyme A (AccCoA) gene
	% Soybean lectin gene
	% Tomato polygalacturonase gene
Screening methods (category 1)	% Beta lactamase gene (bla) coding for ampicillin resistance in prokaryotes
	% CaMV promoter (P-35S)
	% CaMV terminator (T-35S)
	% *Agrobacterium tumefaciens* nopaline synthase terminator (T-Nos)
	% Neomycin-3'-phosphotransferase II (*nptII*) coding for neomycin/kanamycin resistance in plants
Gene-specific methods (category 2)	% *bar* (phosphinotricin acetyltransferase) gene
	% Cry1A(b) gene (synthetic)
Construct-specific methods (category 3, all junctions)	% Bt11 maize: IVS6 – Cry1A(b) gene
	% Bt176 maize: CDPK promoter-synthetic Cry1A(b) gene
	% DLL25 maize: Tr7 (complement) – P35S
	% GA21 maize: OTP-EPSPS gene (RoundupReady tolerance)
	% Mon802: hsp 70 intron 1 – CP4 EPSPS gene
	% Mon810 maize P35S– hsp 70 intron 1; hsp 70 intron-Cry1A(b) gene
	% 40-3-2 (Roundup Ready) soybean: P35S - CTP
	% T25 maize: pat phospinotricin acetyltransferase) gene – T35S
	% Zeneca tomato: TNos – truncated tomato polygalacturonase gene
Event-specific methods (category 4, all junction between host plant genome and integrated recombinant DNA)	% Bt11 maize
	% Mon810 maize
	% CBH-351 (Starlink) maize
	% Roundup Ready soybean

(Adapted from Holst-Jensen *et al.*, 2003)

identify the GMO conclusively since the presence of one of the screening targets does not necessarily imply the presence of GMO-derived DNA. The sources of P-35S or T-35S could be naturally occurring CaMV (Wolf *et al.*, 2000) and it is generally believed that *Agrobacterium* or other soil bacteria containing one or more of the targets are present in soil.

It should be noted though, that the natural prevalence of the targets found in GMOs has not been carefully assessed (K. Nielsen, University of Tromso, Norway,

2002, personal communication). An additional source of uncertainty may be the presence of cloning vector DNA in the DNA polymerase, for example AmpliTaq (Applied Biosystems) contains amplifiable *bla* DNA.

b) Category 2, gene-specific methods
The gene of interest may also be of a natural origin, but is often slightly modified, for example by truncation or altered codon usage (Hemmer, 1997).

c) Category 3, construct-specific methods
These methods target junctions between adjacent elements of the gene construct, for example between the promoter and the gene of interest. With these methods, a positive signal will only appear in the presence of GM-derived material. Compared to gene-specific methods, there is a higher possibility to identify the GM source of the DNA.

d) Category 4, event-specific methods
The only unique signature of a transformation event (within the limitations of current technology) is the junction at the integration locus between the recipient genome and the inserted DNA. This junction is the target of event-specific methods.

For example, an event-specific identification method using real-time PCR for StarLink maize (event CBH-351) has been developed. This method proposed the detection of an internal detection site in the cry9c coding region, as well as two event-specific target sites at the junction between the CBH-351 insert DNA and the genomic plant DNA (Windels *et al.*, 2003).

Confirmatory Assays in GMO Testing

Confirmation or verification of the identity of the amplicon is necessary to assure that the amplified DNA really corresponds to the chosen target sequence and is not a by-product of unspecified binding of the primers. Several methods are available for this purpose (Bonfini *et al.*, 2001):

a) Gel electrophoresis is the simplest approach to ensure if the PCR products have the expected size. However, there is a risk that an artifact having the same size of the target sequence may have been amplified. Therefore, the PCR products should be further verified for their restriction endonuclease profile (Meyer, 1995).

b) Even more reliable is a Southern blot assay, whereby the amplicon is separated by gel electrophoresis, transferred onto a membrane and hybridized to a specific DNA probe.

c) Another possible control is to subject the PCR product to a second round of PCR cycle in a technique that is called nested PCR (Koppel *et al.*, 1997). Here, two different sets of primers – an outer and an inner (which is also called a nested pair) pair — are used within the target region in two consecutive rounds of PCR amplifications. This strategy substantially reduces the problem of unspecific amplification, as the probability for the inner pair of primers of finding complementary sequences within the non-specific amplification products of the outer pair is extremely low.

d) The most reliable way to confirm the authenticity of a PCR product is by sequencing (Hupfer *et al.*, 1997).

PCR Methods for GMO Quantification

The purpose of quantification is to determine the amount of one or more GMOs in a product or seed lot which will enable a laboratory to assess compliance with the threshold regulation. If a product has been shown to contain (one or more) authorized GMOs, then it becomes necessary to assess compliance with

the labeling threshold regulation through determination of the amount of each of the GMOs present in the individual ingredients from which it has been prepared (e.g. the maize flour).

Two things are important for DNA-based quantification of GMOs (Taverniers *et al.*, 2004): (a) the way in which the relative GMO percentage is determined, and (b) the type of calibrators used. Absolute quantification results/values are derived from tests that measure the amount of a substance, e.g. how many milligrams of a specific protein or how many copy numbers of a specific DNA sequence are present. The larger the size of the sample tested, the higher the absolute quantity (Griffiths *et al.*, 2003).

Relative quantification results/values are derived from tests that measure the amount of a substance relative to another substance, e.g. how many milligrams of a specific protein are present per gram of total protein or how many copy numbers of a specific DNA sequence are present per genome. Results are expressed as a percentage. This percentage does not change with an increase in the size of the sample being tested. For compliance to the GMO labeling legislation, relative quantification is required. Protein-based methods are generally not suitable for determining the relative amount of an ingredient that is genetically modified unless it is the sole ingredient in the sample and a suitable reference material is available (Griffiths *et al.*, 2003).

For relative GMO concentration in food mixtures, the quantification of a GM-marker has to be normalized to a plant-specific reference gene (Hupfer *et al.*, 2000). In practice, accurate relative quantification might be achieved by a combination of two absolute quantification reactions: one for the GMO-specific gene and a second for a plant reference gene. With the assumption that GMO material has been submitted to the same treatment as the non-GMO material, the measurement can be expressed as percentage genome/genome (% g/g) or percentage weight/weight (% w/w) (Bonfini *et al.*, 2001).

Competitive PCR

The first quantitative PCR tests were based on competitive PCR. Quantification is done by comparison of the amount of the end product (end point quantification), that is when the PCR is completed (Holst-Jensen *et al.*, 2003). By reducing the influence of the varying amplification efficiency, the accuracy of the quantitative information obtained by PCR can be improved with competitive PCR. Here two target sequences with very similar features and amplifiability are co-amplified in a single reaction tube to correct for the decrease in reaction efficiency. Because the two targets compete for available nucleotides, primers and DNA polymerase, the relative quantity of end product is assumed to correspond to the relative quantity at the beginning of the first PCR cycle (Wiseman, 2002).

In this PCR, a known amount of internal standard is added to each reaction. The same primers will amplify both the DNA sequence present in the specific GMO and in the internal standard. This form of PCR involves the effective titration of a set amount of internal standard with different amounts of target DNA. Both the single-competitive and double- competitive PCR are available for this technique.

Competitive PCR requires development of suitable competitor molecules and is highly sensitive to the starting concentrations and dilution of template DNA. It involves massive pipetting of amplified DNA and visualization by agarose gel electrophoresis, which is associated with a significant risk of cross-contamination. Therefore it is gradually being replaced with more sophisticated alternatives, in particular real-time PCR (Holst-Jensen *et al.*, 2003).

Real-Time PCR

Real-time PCR is the technique of choice for nucleic acid quantification, and may also be

used for qualitative purposes. In the field of detection of GMOs, quantification of biotechnology products may be required to fulfill legislative requirements. Successful quantification depends crucially on the quality of the sample DNA analyzed. The crucial influence of the extraction technique and sample matrix properties on the results of GMO quantification has been demonstrated in the study by Cankar *et al.* (2006). The extraction methods must ensure high yield and quality of the DNA obtained and must be carefully selected, since even components of DNA extraction solutions can influence PCR. GMO quantification is based on a standard curve, therefore similarity of PCR efficiency for the sample and standard reference material is prerequisite for exact quantification (Cankar *et al.* (2006).

Real-time PCR is rapidly gaining popularity due to the introduction of several complete real-time PCR instruments and easy-to-use PCR assays. Real-time PCR allows the monitoring of the amplification reaction during amplification (in real-time) in a closed environment without interfering with the reaction. Fluorescence signal corresponding to an increased amount of amplification product can be measured and visualized on a computer screen. Software can immediately convert the signal into quantitative estimates. This speeds up quantification, and the risk of cross contamination is low (Holst-Jensen *et al.*, 2003).

The number of PCR cycles necessary to generate a signal that is significantly and statistically above noise level is taken as a quantitative measure and is called cycle threshold (Ct). As long as the Ct value is measured at the stage of the PCR where the efficiency is still constant, the Ct value is inversely proportional to the log of the initial amount of target molecules (Bonfini *et al.*, 2001).

A recent EU commission recommendation (2004/787/EC) states that the quality level of a seed lot or other plant propagating material and the associated statistical uncertainty are defined in relation to threshold from GMOs and related to the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes. The results of quantitative analysis should be expressed in the same way as mentioned above. However, the CRMs that are currently available have not been certified on the basis of copy numbers and hence inaccuracies lie in the conversion of the GM content in weight/weight to copy numbers. Plasmid calibrants certified on the basis of copy numbers may therefore be needed to fulfill this new definition of GM percentage. The demonstration on the applicability of using plasmid DNA as opposed to genomic DNA for quantitative PCR calibration is therefore of particular importance with regard to future CRMs for GM quantification.

Most commonly, GMO quantification by real-time PCR method is calculated from the ratio of the target transgenic specific DNA sequence copy number versus the DNA sequence copy number of the respective target plant species (taxon gene sequence). Determination of the copy number by real-time PCR methods involves the establishment of calibration curves based on analysis of a set of calibrators such as genomic DNA or plasmid DNA. Plasmid DNA markers containing a cloned transgenic sequence have been used and are increasingly being promoted as the reference material (Toyota *et al.*, 2006).

Most of the established analytical methods for GMO quantification are based on PCR due to its sensitivity, specificity and applicability to the analysis of complex food matrices. Many real-time PCR systems are based on fluorescent detection such as TaqMan chemistry, which have been developed to identify and quantify GM soybeans, GM maize and GM varieties of other agricultural commodities (Toyota *et al.*, 2006).

Real-time PCR systems using TaqMan chemistry are based on the use of fluorescent TaqMan probe that monitors the formation of the PCR product during each cycle of the

reaction. Examples of systems used for on-line quantification are the LightCycler System and the Applied Biosystem (ABI), to mention a few. Two approaches are used: the fluorescent intercalator SYBR Green 1 and specific hybridization probes (Toyota *et al.*, 2006).

Two methods are available for deriving a relative percentage of GMO (Taverniers *et al.*, 2004): (a) the 'delta Ct methods''– both Ct values are directly compared to each other. The difference between the Ct values is used for directly calculating the GMO content, and (b) the 'standard curve method''– a standard curve is set up for each target, expressed in absolute numbers of haploid genome copies. Comparison of copy numbers results in a percentage (Holst-Jensen *et al.*, 2003). The advantage of standard curve quantification is that Ct values are compared only with Ct values of the same amplicon. So the final quantitative estimate is based on comparing an estimated quantity of GM to the estimated quantity of reference. The estimate is therefore the ratio of quantity to quantity, not of PCR cycle to PCR cycle.

Reference Targets: Species or Taxon Specific

For quantification, the copy number of the GM target relative to genome copies of the corresponding species is important because the principle of quantification of GM content is to compare the relative ratio of these two. In this case, PCR methods are needed for the reference target as well as GM-specific target (Holst-Jensen *et al.*, 2003).

In a diploid organism, each cell contains two copies of a single copy gene, while a tetraploid contains four copies per cell. If the GM-specific target is inserted in a single copy, then quantification becomes relatively simple. For example in Roundup Ready soybean, the full-length gene construct is inserted in a single copy, and through backcrossing the diploid GMO has been made homozygous. Thus, in this case, each GM cell has a 1:1 ratio of the GM target and the lectin gene (Holst-Jensen *et al.*, 2003). However, if the GM-specific target is inserted in several copies, quantification becomes more uncertain, in particular if the exact number of inserted copies is unknown. Heretozygosity and ploidy level can introduce additional uncertainty, and testing on single plants, tissues or kernels/seeds/grains may be required to control these parameters (Holst-Jensen *et al.*, 2003).

General Advantages of DNA Detection Methods

DNA detection methods offer certain advantages (Griffiths *et al.*, 2003) as follows:

a) these methods are suitable for a range of applications from screening methods to event-specific methods.
b) DNA composition is the same in all cells of an organism. As such, any part of a plant can be used to detect for GMO presence provided that the DNA can be efficiently extracted.
c) DNA-based assays provide relative quantification as a percentage measurement as required by labeling legislation.
d) DNA-based methods are versatile and sensitive. If relevant target sequences are known, the necessary primers may be designed. Using the same basic approach, a new and completely different GMO may be detected. In comparison, protein methods require prolonged procedures of antibody development.

General Disadvantages of DNA Detection Methods

However, certain disadvantages (Griffiths *et al.*, 2003) also exist as follows:

a) DNA-based methods require skilled analysts because these methods tend to use high-end instruments.
b) these methods can be expensive, time consuming and may be unsuitable for on-site testing.
c) some processing procedures such as excessive heat, ultraviolet light, acidic conditions and nuclease activity can damage or remove DNA.
d) some food ingredients such as proteins, fats, and polysaccharides will inhibit DNA

amplification and thus prevent detection of DNA.

e) these methods are susceptible to cross-contamination and carry-over from previous PCRs as PCR is an amplification method. To ensure accuracy of the results, a series of positive and negative controls are run.

f) not all DNA sequences, primer information and certified reference materials are available to design DNA-based methods.

Real-Time PCR versus End-Point PCR

For real-time PCR, the level of fluorescence is monitored during each cycle (Griffiths *et al.*, 2003). The intensity of fluorescence is directly related to the amount of amplified product. In this case, detection is carried out in the same tube as the PCR. A range of fluorescent chemistries is available for detection of amplified products during real-time PCR. The following detection systems lend themselves to high-throughput screening and automation.

a) SYBR Green – which is a dye that fluoresces when bound to double strand DNA. Detection using this dye is not suitable for doing, in the same tube, more than an amplification (multiplexing) as it binds to all double-stranded DNA in a non-specific manner.

b) Melting curve analysis – this analysis can be used in conjunction with SYBR Green detection to confirm that the melting curve characteristics corresponds to those of the expected PCR product.

c) Probes – detection methods using probes are much more specific than those using SYBR Green leading to improved sensitivity of detection. Probes that are currently available for GMO testing are Taqman probes, FRET (Fluorescence Resonance Energy Transfer) probes and Molecular Beacons probes.

When DNA is amplified, the product can be detected and quantified by monitoring an increase in DNA associated fluorescence at the end of a defined number of cycles and this is known as end-point PCR (Griffiths *et al.*, 2003). In this case, the sample must be taken out of the tube for detection. For end-point PCR, a variety of methods can be used to detect and verify the PCR product following amplification, such as: gel electrophoresis, gel electrophoresis followed by blotting onto a membrane, nested PCR, sequencing, mass spectrometry, surface plasmon resonance and biosensors, capillary electrophoresis, microarray analysis and micro-fluidic systems. The advantages of real-time PCR are firstly, the reduced time needed for individual tests and secondly, real-time PCR significantly reduces the risk of carryover contamination in the laboratory because there is no need to analyze the PCR product on the agarose gel as in end-point PCR (Kok *et al.*, 2002).

Calibrators for Real-Time PCR

The quality of the analytical measurement data obtained by quantitative real-time PCR depends on the correct use of calibrators and reference materials (RMs). Most GMO methods use genomic DNA (gDNA) solutions, derived from powdery certified reference material (CRM) produced by the Institute for Reference Materials and Measurements (IRMM, Geel Belgium). The powder CRMs as such, are matrix RMs. As an example, Table 4 displays the current list of GMO CRMs available from IRMM. There also exists 100% pure GMO material such as seeds, leaves or grains. Besides IRMM, examples of other sources of reference material are Bayer CropScience and American Oil Chemists Society (AOCS), to mention a few.

Due to lack of pure RMs, solutions of DNA isolated from powder CRMs are used as calibrators. Since these CRMs are made by mixing GM seeds with non-GM seeds in certain concentrations, a relative percentage of GMO in this case represents a weight/weight percentage. As percentages based on weights are not exactly the same as those based on genome copies, the suitability of these CRMs

Table 4
List of Certified Reference Materials available

Crop material	GMO product
Soybean	% Roundup Ready
Maize	% Bt-176
	% Bt-11
	% MON 810
	% MON 863
	% NK 603
	% GA21
	% 59122
	% MIR604
	% 1507
	% MON 863 x MON 810
Potato	% EH92-527-1
Sugar beet	% H7-1
Cotton seed	% 281-24-236 x 3006-210-23

(*Source*: IRMM)

for the estimation of DNA copy number may be doubtful.

In answer to the growing need for alternative types of calibrators for GMOs, studies on the use of plasmid DNA calibrators have been reported (Taverniers *et al.*, 2004; Burns *et al.*, 2006). In the study by Burns *et al.* (2006), results demonstrated that the specific plasmid DNA used in the inter-laboratory trial provided a suitable alternative to genomic DNA for use as a calibrant in GMO quantification. The study reported that plasmid calibrants gave equal or better performance characteristics in terms of precision and closeness to the expected value, than their genomic equivalents. With regard to the ease of production, storage, distribution, high stability and its performance, plasmid DNA calibrators may be preferred over genomic DNA calibrators in the future.

One drawback of plasmid DNA calibrators is that they, as such, only contain the pure analyte and are not similar to real samples of interest. This may be overcome by 'matrixmatching', which is by spiking the plasmid DNA RMs in a background of genomic DNA as done in a study by Taverniers *et al.* (2004).

Another prerequisite for delta Ct methods is that equal PCR amplification efficiencies for both targets are obtained. To overcome this drawback, Taverniers *et al.* (2004) successfully developed a duplex quantification method with plasmid DNA calibrators expressed in copy numbers. Their study demonstrated that plasmid DNA molecules containing either one or multiple target sequences form perfect alternative calibrators for GMO quantification and are especially suitable for duplex PCR reactions.

PROTEIN ANALYSIS METHODS

The specific detection of a novel protein synthesized by a gene introduced during transformation constitutes an alternative approach for the identification of GM crops and products. Genetic modification is not always specifically directed at the production of a new protein and does not always result in protein expression levels sufficient for detection purposes. In addition, certain proteins may be expressed only in specific parts of the plant or expressed at different levels in distinct parts or during different phases of physiological development (Bonfini *et al.*, 2001).

Immunoassays

Protein detection methods are based mainly on immunoassays. Immunoassays are analytical measurement systems that use antibodies as test reagents. Antibodies are proteins produced in the serum of animals in response to foreign substances (antigens) and specifically bind the substance that elicited their production. In case of GMOs detection, the antigen can be the newly synthesized protein. The two most common test formats are enzyme-linked immunosorbent assay (ELISA) and immunochromatographic lateral flow strip tests (Bonfini *et al.*, 2001).

ELISA

ELISAs have been designed to detect and semi-quantify a novel GM protein or trait. To date, several ELISA methods have been developed that are specific for gene products widely expressed in GM plants such as the neomycin phosphotransferase II (nptII) gene product, the enzyme 5-pyruvylshikimate-3-phosphate synthase (EPSPS), the *Bacillus thuringiensis* (Bt) insecticide Cry1Ab, and herbicide-tolerant phosphinotricin acetyltransferase (PAT) protein. ELISA tests are not event-specific (Bonfini'*et al.*, 2001).

Currently, two major traits (insect resistance and herbicide tolerance) have been engineered into four major crops: soybean, maize, cotton and canola. Protection from insects has been effected through the use of specific genes isolated from the naturally occurring soil bacterium *B. thuringiensis*. These genes cause the production of specific insecticidal proteins known as Cry proteins. The major use of Bt Cry genes for this purpose currently is in corn and cotton. Although there are commercial varieties of corn expressing the Cry1Ab, Cry1Ac and Cry9C proteins, most commercial acreage of biotech maize expresses Cry1Ab. Another maize event expresses the PAT protein, which confers tolerance to the herbicide glufosinate (Stave, 2002).

Commercial immunoassay methods are currently available for detection and quantification of GM crops expressing Cry1Ab, Cry1Ac, Cry3A, Cry2A, Cry9C, CP4 EPSPS and PAT protein. Compared to PCR methods, only two ELISA methods; which are for the detection of the CP4 EPSPS protein in Roundup Ready soybeans and the detection of the Cry1Ab protein in MON810, have been the subject of large international collaborative studies designed to assess whether such methods can be used to determine the concentration of GM ingredients in samples of ground grain (Stave, 2002).

Lateral Flow Strips

Lateral flow strip technology is a variation of ELISA with antibodies that are immobilized onto a test strip in specific zones. The purpose of the flow strips is to provide a rapid test for the detection of GMOs. The test can be performed with a kit and does not require any major equipment. These strips are suitable for on-site use, with minimal training required. Sample preparation simply involves crushing the sample and mixing it with protein extraction solutions provided in the kit (Griffiths *et al.*, 2003).

General Advantages of Protein Detection Methods

Protein detection methods offer certain advantages (Griffiths *et al.*, 2003) as follows:

a) economical compared to DNA testing methods
b) less skill required by personal compared to DNA testing methods
c) equipment setup is cheaper compared to DNA testing methods
d) moderate sample preparation
e) relatively fast assay
f) offers qualitative and semi-quantitative formats
g) it is robust and has simple assay formats
h) it is suitable and cost-effective for batch analysis of samples
i) can be used on-site for GM grains verification

General Disadvantages of Protein Detection Methods

However, certain disadvantages (Griffiths *et al.*, 2003) also exist as follows:

a) these methods are generally less sensitive than DNA detection methods.
b) laboratory production of antibodies is a slow and difficult task that requires a great deal of skill and experience.
c) recognition of the protein by the antibody is one of the main limitations because antibody binding relies on shape, so the target protein must be correctly folded in order to be recognized by the antibody.

d) protein methods are less suited for general GMO screening as a single antibody will only recognize one particular protein and not various events that might have the same protein.
e) protein levels are not the same in all cells, and can also vary at different stages of the cell's life cycle.
f) protein assays produce an absolute rather than relative quantification.
g) false positives may occur due to cross-reaction with other components in the sample.
h) some GMOs do not express detectable levels of the target protein.
i) there may be limited or no expression of the novel protein in the plant tissue.

CONCLUSION

The genetic code is universal. It means that genetic information is always borne by DNA whatever the organism (bacteria, fungus, plant or animal). Thus, DNA is easily recombined and transferred from one organism to another. Furthermore, DNA is a ubiquitous molecule as all the cells that form an organism contain the same DNA. In addition, DNA is a resistant molecule, in the sense that it is quite resistant to variation in heat and acidity. All these properties represent advantages for the detection of this molecule in various agriculture products, such as food and animal feed. Finally, very low amounts of DNA may be amplified using PCR technique, which allows easy identification.

Thus, the PCR technique can target genes introduced into the genetically engineered plants because of a set of primers that amplify sequences from these cloned genes or from regulatory sequences linked to them. DNA fragments amplified by PCR and having the expected size, are the signature of the sample which is being analysed. They indicate whether products are made from genetically modified or conventional plants. This enables the determination, in case of a positive result, with which genetically engineered species the products are made.

The primary question here is how to determine the most appropriate method for detecting GMOs in a particular application. Unfortunately, it is not possible to derive a single table, listing types of samples and the appropriate testing method. The range of sample types, from raw commodities to highly processed food, is extensive and the reasons for GMO testing are diverse. In addition, the number of varieties of GMOs grown commercially increases each year. Due to these factors, and many others, each sample must be assessed on a case-by-case basis to determine the most appropriate testing method.

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