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REVIEW

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Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant-derived food products

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Abstract Numerous analytical methods, both qualitative and quantitative, have been developed to determine reliably the presence and/or the amount of genetically modified organisms (GMOs) in agricultural commodities, in raw agricultural materials and in processed and refined ingredients. In addition to the "classical" methods for DNA and protein analysis, e.g. polymerase chain reaction and enzyme linked immunosorbent analysis, certain types of GMO-containing matrices can be profiled by complementary chemical analysis methods such as chromatography and near infrared spectroscopy. This review summarises the status of the most widely used GMO analysis technologies, identifies new areas of analytical investigation and discusses current needs and future challenges.

Keywords Genetically modified organisms · Polymerase chain reaction · Enzyme-linked immunosorbent analysis · Chromatography · Near infrared spectroscopy

Introduction

Agricultural biotechnology has opened new avenues in the development of plants for the production of food, feed, fibre, forest and other products. In the few years since the first commercial introduction of a genetically modified organism (GMO), the cultivation of several transgenic crop species has grown rapidly to more than 40 million ha worldwide [1], i.e. approximately 4% of the total world acreage.

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GMOs can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating or natural recombination, i.e. by being genetically modified (GM) or by recombinant DNA technology. The addition of foreign genes has often been used in plants to produce novel proteins that confer pest and disease tolerance and, more recently, to improve the chemical profile of the processed product, e.g. vegetable oils. In the European Union (EU) and other regions, the use of this technology, the consequent release of GMOs in the environment and the marketing of GMO-derived food products are strictly regulated[1] [2, 3, 4, 5, 6].

The legal provisions are different for GMOs (i.e. transgenic organisms that have replicative capacity) and for GM-containing foods. One example of such a discrepancy is the existence of a *de minimis* threshold for food labelling (see later) that does not have its counterpart, for instance in the labelling of GM grains. The so-called "threshold regulation" specifies that foodstuffs must be subject to labelling where material derived from these GMOs is present in food ingredients in a proportion above 1% of the food ingredients individually considered [5]. That limit is set at the level of ingredients and therefore, if a final product scores positive after a screening method, its respective ingredients should be quantitatively assayed to assess if it contains less or more than 1% GMO. Detection of DNA and/or proteins might become difficult when processed and highly refined ingredients, such as starch, sugar or vegetable oils,

[1] EU biotechnology legislation is mainly based on Directive 2001/18/EEC on the deliberate release into the environment of GMOs [2], as well as on Regulation 258/97/EEC on novel foods and novel food ingredients [3] and Regulation 1139/98/EEC concerning the compulsory indication of the labelling of two GMO products [Roundup Ready soybeans and Bt-176 maize)] [4] that had been placed on the market before the Novel Foods Regulation came into force. Recently, the labelling Regulation has been amended by Regulation 49/2000/EEC setting a 1% threshold for labelling requirements [5] and by Regulation 50/2000/EEC that demands labelling of additives and flavourings derived from GMOs [6].

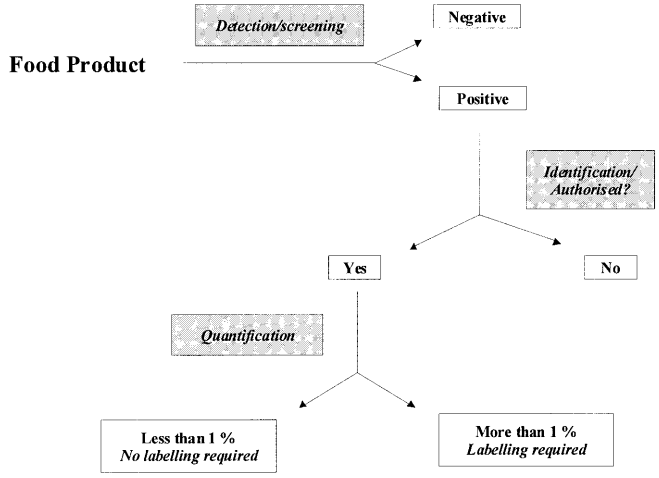

Fig. 1 Operational procedures for detection, identification and quantification of genetically modified organisms to comply with the labelling regulation in the European Union

are considered. A food product might even have undergone such extensive processing that ingredients of potential GMO origin are no longer detectable.

The concept of the so-called negative list has been introduced in the Novel Foods Regulation [3] to deal with highly processed foods in which no traces of DNA and/or protein can be found. The argument followed is that for those samples, no analytical methods exist to distinguish between foods derived from GMOs and foods derived from non-GMOs and consequently, those should be exempted from labelling requirement. The concept and content of such a negative list are currently under discussion among experts [7, 8]. If such a list were established, the listed products would no longer be subject to analytical testing and monitoring.

The need to monitor and verify the presence and the amount of GMOs in agricultural crops and in products derived thereof has generated a demand for analytical methods capable of detecting, identifying and quantifying either the DNA introduced or the protein(s) expressed in transgenic plants, because these components are considered fundamental constituents [9, 10, 11, 12, 13, 14, 15]. In addition, for certain types of GM food such as vegetable oils with altered fatty acid profiles, chemical analysis, such as chromatography and near infrared spectroscopy, may be a complementary or alternative tool for GMO detection.

Other aspects that should be taken into consideration are plant breeding processes and biological variability and their influence on the quantification of GMOs. One example is the need for a method that could distinguish between two independent but related GMOs and one simple GMO in which both traits have been combined (the so-called issue of stacking genes by breeding).

Methods to verify compliance with labelling regulations are mainly based on the detection of specific DNA fragments, e.g. by polymerase chain reaction (PCR) or on the detection of newly expressed proteins, e.g. by enzyme-linked immunosorbent analysis (ELISA). Figure 1 depicts a general outline of the different procedures to assay sampled food for the presence of GMOs.

In general this process consists of three different steps:

1. Detection (screening of GMOs) in order to gain a first insight into the composition of the food and agricultural product. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results in all control laboratories, which can be achieved through inter-laboratory validation.
2. Identification to reveal how many GMOs are present, and if so, whether they are authorised within the EU (or other countries with regard to their regulations). A prerequisite for the identification of GMOs is the availability of detailed information on their molecular make-up. Molecular registers that, along with the scientific data, contain the tools for control authorities to design appropriate identification methods, are essential to fulfill this task. The European Commission's Joint Research Centre is in the process of setting up such a database, which will be used by the National Competent Authorities.
3. Quantification, in order to determine the amount of one or more authorised GMOs in a product or seed lot, and to assess compliance with the threshold regulation. For this approach it is necessary to get a better understanding of DNA/protein degradation during processing and of the robustness of the analytical methods.

Although much progress has been made in the development of genetic analysis methods, such as those based on the use of PCR, several other analytical technologies that can provide solutions to current technical issues in GMO analysis, e.g. DNA chip technology and mass spectrometry, are emerging. This review examines the different steps that are required for GMO analysis and summarises the status of most widely used GMO analysis technologies. Furthermore, it identifies new areas of analytical investigation and discusses current needs and future challenges.

Sampling and sample preparation

The sampling procedure determines the "representativity" of a result, whereas quality and quantity of analytes may vary depending on the sample preparation. Sampling and sample preparation are thus crucial steps in the process of GMO detection [16]. The limit of detection of the analytical method as a whole is determined, not by the most sensitive part of the procedure, but by the least. In most cases this is the sample size.

Sampling – sample size, homogeneity of the sample and threshold limit

One of the major problems in analytical testing is the sampling procedure. A sample has to be representative

of the batch/lot of the product from which it was taken, while 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. Therefore, on the one hand, the sampling plan must ensure that the field sample is taken in a statistically representative manner with respect to the larger lot of material and, especially, to its state of homogeneity (or rather heterogeneity). On the other hand, the sample size must be sufficiently large to allow reliable detection at the desired sensitivity. Sample representativity must be maintained during subsequent reduction of the field sample to laboratory and test samples.

The expected variance of the sample (often described as heterogeneity), and thus the applied sampling plan, depends on the type of material to be analysed.

1. Raw materials are often not systematically mixed during harvest, storage, etc., resulting in strata that can seriously invalidate assumptions associated with simple random sampling.
2. Ingredients are processed and thus already present a restricted degree of variance, while different batches may also present different characteristics.
3. Processed foods should contain GM material only as a source of one or several of various ingredients, so that a potentially strongly stratified variance distribution can be expected in many cases. However, within each ingredient of the processed food, variance should be low.

The degree of heterogeneity of a given sample and the actual threshold limit, which is set for acceptance of the presence of GM material, will define both the number of samples to be taken and the appropriate sample size. The higher the degree of heterogeneity, the more critical will be the choice of the appropriate sampling plan. Moreover, when only low levels of GMO material are acceptable, the required sample size will increase accordingly in order to be representative. For the sampling of food commodities, there is already experience with well-tested sampling plans for analogous detection problems [17, 18, 19, 20].

In Europe as well as in the United States the demand for sampling guidelines in GMO testing has been acknowledged. The United States Department of Agriculture (USDA), Grain Inspection, Packers and Stockyards Administration (GIPSA, Washington, D.C.) has recently established sampling guidelines for diagnostic testing for GM grains [21, 22]. For example, on the basis of the formula (designed for a single-step sampling procedure and qualitative analytical testing):

$$n = \log(1-(G/100))/\log(1-(P/100))$$

where n is the sample size (number of kernels), G is the probability of rejecting a lot concentration, and P is percent concentration in the lot, the sample size should amount to 299 kernels or beans in order to obtain a 95% probability of rejecting a lot with 1% concentration of GMO, i.e. a "buyer's risk" of 5% to accept a lot with more than 1% GMO content [22]. If the threshold limit was set at 0.5% GMO at a 95% probability of rejection, the size of the field sample would need to be increased up to 598 kernels. However, at a sample size of 299 kernels the "seller's risk" of having a lot rejected, which contains only 0.5% GMO, is still about 78%. Therefore, in order to provide means of controlling marketing risks for both buyer and seller, multiple sampling plans for qualitative analytical testing have been developed [22, 23, 24]. A multiple sampling plan is defined by the number of samples to be taken and tested, by the maximum number of positive results allowed for the lot to be acceptable and by the number of kernels in each sample. Buyer and seller have to agree on these three values, and thereby determine the marketing risk both of them are willing to take [22].

Two other references applied to the quantitative analytical testing of the proportion of GMO grains in a lot describe a different sample size:

1. The Working group "Genetically Modified Foodstuffs" within the Technical Committee CEN/TC 275 of the European Committee for Standardization (CEN, Brussels, Belgium) assumed that a sample size of 10,000 "particles" leads to a relative sampling error of less than 20%, if the examined lot contains 1% GMO [25].
2. Hübner and co-workers [26] calculated that for a homogeneous distribution a sample size of at least 3500 particles should be examined for an expected GMO-content of 1% in order to establish a result with 95% confidence and a relative sampling error lower than 20% (corresponding to a coefficient of variation of 20%). In the case of "heterogeneously distributed GMO particles" the sample size increases to 10,000 particles.

The different sample sizes here reflect different requirements for qualitative and quantitative GMO testing.

Sample preparation – extraction and purification of the analytes

As DNA is a rather stable molecule and the most common DNA detection method (PCR) is very sensitive, DNA is the preferred analyte for almost any kind of sample (raw materials, ingredients, processed foods). Provided that the laboratory sample is representative for the field sample and that it has been adequately homogenised, even small aliquots of vegetal material are sufficient for DNA extraction, usually between 100 mg [27] and 350 mg [28]. Failures in extracting detectable DNA levels have so far been reported for soybean sauce and refined soybean oil [29, 30], as well as for distilled ethanol produced from GM potatoes [31]. However, method optimisation and the use of large volumes has allowed [32] the extraction of DNA from cold-pressed as well as from refined rapeseed oil, although this DNA could not be unequivocally identified. Pauli and co-workers [33] recent-

Table 1 DNA isolation methods used for the detection of GMOs in plant material and plant-derived food products. *PCR* Polymerase chain reaction, *CTAB*, cetyltrimethylammonium bromide, *SDS* sodium dodecyl sulphate + successful PCR amplification, - unsuccessful PCR amplification

Method	DNA quality	DNA yield	PCR	Material	Reference
Wizard method			+	Soybean powder, maize powder	[36]
			+	Bruised soya grain, lecithin	[37]
			+	Soya leaves	[38]
	High	Low	+	Soya products (tofu, flour, lecithin)	[28]
			–	Soybean oil	[30]
			+	Foodstuffs (55) derived from soybean, corn, rice, sugar beet, tomato and wheat	[33]
CTAB method			+	Raw potato	[39]
			+	Raw soybeans	[40]
			+	Raw tomato	[41]
	High	Low	+	Soya products (tofu, flour, Lecithin)	[28]
			+	Foodstuffs derived from potato	[33]
CTAB method with QIAquick column			+	Soya products: meal, oil, lecithin, tofu, chocolate, etc.	[29]
			–	Soya sauce, refined soya oil	
CTAB method with Nucleon Phytopure			+	Maize, potato, soya, sugar-beet, tomato	[27]
Nucleon Phytopure method	High	Low	+	Soya products: tofu, flour, lecithin	[28]
Qiagen DNeasy method					
Chelex 100 method	Low	High	+		
Alkali method					
AlkaliX method					
ROSE method					
ROSEX method					
Dellaporta method			+	Maize grains	[42, 43]
Hexane/guanidine thiocyanate with gel filtration			+	Raw and purified lecithin	[44]
CTAB method with High Pure PCR Template Preparation Kit (Boehringer)			+	Bt-maize in silage	[45]
Modified QIAamp DNA Stool Mini Kit			+	Cacao-derived products	[46]
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	[47]

ly reported detection of DNA extracted from a large variety of food products and processing stages, although no DNA could be extracted from refined sugar and oil.

Protein detection methods require proteins with an intact tertiary or quaternary structure since they are based on immunoassays, or the comparison of protein patterns in 1- or 2-dimensional gel electrophoresis. Protein detection is, therefore, limited to fresh and unprocessed foods.

DNA isolation methods

The efficiency of the PCR, as with any other DNA assay, depends on DNA quality and purity. DNA quality is determined by its fragment length and its degree of damage due to the exposure to heat, low pH and/or nucleases that cause hydrolysis, depurination and/or enzymatic degradation. Therefore, DNA quality varies according to the material under examination, the degree of processing the sample has been subjected to and the DNA extraction method applied. It is important to keep in mind that DNA isolated from processed foods and certain agricultural materials such as cured tobacco leaf is of low quality, with available target sequences being rather short, e.g. 100–400 bp for soybean protein preparations and processed tomato products [12]. Thus, an appropriate choice of primers in order to obtain short amplicons should be made.

The purity of DNA can be severely affected by various contaminants in food matrices [34]. Contaminants may be substances originating from the material under examination, e.g. polysaccharides, lipids and polyphenols [11, 35] or chemicals used during the DNA extraction procedure, e.g. as reported for cetyltrimethylammonium bromide (CTAB), or hexadecyltrimethyl ammonium bromide, ROSE and the alkali method [28]. For example, *Taq* polymerase, the key enzyme used in the PCR reaction, is inhibited by polysaccharides, ethylenedi-

Table 2 DNA isolation methods used for molecular marker analysis (AFLP, RAPD, PCR-RFLP) in plant material and plant-derived food products

Method	DNA quality	DNA yield	PCR	Material	Reference
NaI method			+	Single-seed *(B. oleracea L.)*	[48]
Modified Mettler's method for a single pollen grain			+	Single pollen (*Fagus sylvatica*)	[49]
Modified CTAB method with activated charcoal			+	Cotton, coffee, rubber tree, cassava, banana	[34]
Modified CTAB method with spermine			+	Woody species: bark, dormant buds, etc.	[50]
Carlson/Qiagen (CTAB method with column)	Good	High	+	Leaves and needles: oak, elm, pine, fir, poplar, maize	[51]
Ziegenhagen-upscaled	Good	Medium	+		
Doyle and Doyle	Medium	Medium	+		
Dellaporta/Qiagen (SDS method with column)	Medium	Low	+		
0.5 N NaOH -"grinding and use"-protocol			+	Young leaves: *A. thaliana*, *B. napus*, tobacco	[52]
Treatment by proteinase K in SDS extraction buffer with grinding			+	Dry seeds	[53]
Modified phenol-chloroform-EtOH-protocol			+	Dry tea	[54]
Modified Nucleon PhytoPure method			+	Cured tobacco leaves (flue-cured, Burley, Oriental tobacco)	[11]
CTAB method			+		
Qiagen DNeasy Plant Mini Kit			+		
Method for separation and accumulation of DNA from oil			+[a]	Rapeseed oil	[32]

[a] DNA from rapeseed oil could not be identified unequivocally

aminetetraacetic acid (EDTA), phenol, sodium dodecylsulphate (SDS), etc.

A vast range of methods is available for DNA isolation and many of them have been evaluated for their applicability to GMO detection in plant material and plant-derived foods (Table 1). In Table 2 we have also included a number of methods that have been applied to very resilient matrices, e.g. bark, dry seeds and pollen for DNA fingerprinting purposes.

In general, DNA extraction from plant material has to accomplish the following steps:

1. The breakage of cell walls is usually achieved by grinding the tissue in dry ice or liquid nitrogen.
2. The disruption of cell membranes is achieved by using a detergent (e.g. CTAB or SDS), which is (as well as EDTA and a buffering salt like Tris-HCl) a necessary component of any DNA extraction buffer.

3a. Inactivation of endogenous nucleases is achieved by the addition of detergents and of EDTA, which binds Mg^{2+}, an obligatory co-factor of many enzymes.

3b. Proteinase K may be added for inactivation and degradation of the proteins, particularly in protocols using DNA-binding silica columns.

4. Separation of inhibitory polysaccharides is possible due to the differential solubility of polysaccharides and DNA in the presence of CTAB.
5. Separation of hydrophobic cell constituents, e.g. lipids and polyphenols is attained by extraction with an organic solvent like chloroform.
6. Finally, the separation from the detergent and concentration of DNA is carried out by alcohol/salt precipitation.

Alternatively, in place of steps 4–6, the separation of DNA from other cell components can be achieved via purification on a DNA-binding silica column [36, 55].

Currently, three different approaches to DNA isolation from plant material and plant-derived products are favoured for GMO detection: the CTAB-method, DNA-binding silica columns (various commercially available kits), and a combination of these two. Although the use of these methods often results in rather low yields, the quality and purity of the DNA is satisfactory in comparison to that obtained with other methods, which yield larger amounts of low quality DNA, e.g. alkali, Chelex100, or ROSE [28].

The CTAB method was originally outlined by Murray and Thompson [56] who could extract purified high molecular weight DNA from plants. The procedure roughly follows the above-mentioned outline (steps 1–6), using CTAB as detergent in the DNA extraction buffer. It appears to be an efficient method for a wide range of plant materials and plant-derived foods, especially due to the good separation of polysaccharides from DNA and is, therefore, part of the official protocols for GMO detection according to the German Food Act LMBG §35 [39, 40, 41].

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 [57]. However, it has been reported that polysaccharides tend to bind to silica columns [26] thus affecting the efficiency of the separation.

DNA analysis methods

Irrespective of a variety of potentially available methods for DNA analysis, only PCR in its different formats has so far found broad application in GMO detection/analysis and is a generally accepted method for regulatory compliance purposes.

PCR – principle and confirmatory assays

The PCR allows the millionfold amplification of a target DNA fragment in a highly sensitive and specific manner. Therefore, two primers (synthetic oligonucleotides) frame the target sequence. Each primer is complementary to either one of the two strands from the double stranded DNA target. Starting from a primer attached to the target sequence, an enzyme (*Taq*-polymerase) can generate a complementary copy of this sequence. This allows the duplication of the target sequence in each reaction cycle. In consecutive reaction cycles the number of target sequences grows exponentially according to the number of cycles.

Confirmation of the identity of a certain amplicon is a necessary step in the PCR analysis in order to ensure that the amplified DNA product actually corresponds to the chosen target sequence and is not a product of non-specific binding of the primers. Several methods are available for this purpose:

1. The simplest approach is to control whether the PCR products have the expected size by gel electrophoresis. However, there is a risk that an artefact of the same size as the target sequence has been amplified. Therefore, the PCR product should at least be additionally verified for its restriction endonuclease profile [27, 58].
2. A reliable but time consuming verification method is a Southern blot assay, whereby the amplicon is separated by gel electrophoresis, transferred onto a membrane and hybridised to a specific DNA probe [39, 40, 41, 59].
3. Nested PCR allows discrimination between specific and non-specific amplification signals. Therefore, the PCR product is re-amplified using another primer pair, located in the inner region of the original target sequence [29, 38].
4. The most reliable way to confirm the authenticity of a PCR product is its sequencing. The disadvantage of this approach is that only a few laboratories are equipped to carry it out for routine analysis. Hence, only a few authors have reported on its use [42, 59, 60].

PCR strategies for GMO screening and identification

Any PCR-based detection strategy depends on a detailed knowledge of the transgenic DNA sequences and of the molecular structure of the GMOs in order to select the appropriate oligonucleotide primers. 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, a GC-rich 3′end, etc., the choice will depend very much on the objective of the PCR analysis.

For routine screening purposes, one should focus on target sequences that are characteristic for the entire respective group to be screened. Genetic control elements such as the cauliflower mosaic virus (CaMV) 35S promoter (*P-35S*) and *Agrobacterium tumefaciens nos* terminator (*nos3′*) are present in many GMOs currently on the market [12]. The first GMO screening method was originally introduced by Swiss and German scientists [27] and is based on the detection of *P-35S* and *nos3′*. However, in the meantime other GMOs have been approved, in which more tissue- and stage-specific as well as non-heterologous regulatory genetic elements have been introduced [61]. Besides, as claimed in Directive 2001/18/EEC [2], it can be expected that selection markers for antibiotic resistance will be avoided in future. Additional target sequences are needed in order to guarantee a complete screening procedure. A further aspect is the choice of primers that allow detection of as many variants as possible of a GMO marker. For example, there are at least eight variants of *P-35S* used in GM crops [12]. It should be stressed, however, that the detection of these GMO markers is only an indication 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.

For unequivocal identification, primer selection has to be based on target sequences that are characteristic for the individual transgenic organism, e.g. the cross-border region between integration site and transformed genetic element of a specific GMO (the so-called “edge fragments”), or specific sequence alterations due to truncated gene versions (i.e. cDNA, or altered codon usage). For example, a combination of screening and gene specific detection methods has been used on *Solanaceous* crops to detect the “universal” GMO markers *P-35S* and *nos3′* and widely used virus resistance genes [62, 63].

In a recent extensive review of GMO detection methods applied to tobacco, the Task Force Genetically Modified Tobacco – Detection Methods of the Cooperation Centre for Scientific Research relative to Tobacco, Paris, France (CORESTA) [9] has reported on the successful application of the above-mentioned PCR strategies to different types of dry tobacco leaves, and has highlighted both the advantages and drawbacks of confirmatory tests such as nested PCR, restriction enzyme digestion of amplicons and the nucleotide sequencing of the detected genetic elements.

Generally, one should try to avoid target sequences that may occur as natural contaminants in the sample,

Table 3 PCR methods in genetically modified organism (*GMO*) detection for maize. *Scr* Screening, *ID* identification

GMO product (company)	Scr/ID	Primer	Amplicon length (bp)	Target gene or genetic element	Target sequence Reference	Reference
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)	ID	cry IA(b)	184	*cryIA(b)* gene (from *Bacillus thuringiensis*)		[42]
		bar	264	*bar* gene (from *Streptomyces hygroscopicus)*		
		35S-bar	365	Crossborder sequence: CaMV-promoter/*bar* gene	[65]	
		ampR	828	*ampR* gene (from Plasmid pUC19 from *E. coli*)		
	Control	ivr1	226	exon 3 of *ivr1* gene (corn-specific single copy gene)	[66]	
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)	ID	Cry01/ Cry02	1914	*cryIA(b)* gene (from *Bacillus thuringiensis*)	[67]	[59]
	Control	TR03/ TR04	137	18S-rDNA (highly conserved sequence)	[68]	
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)	ID	Cry01/ Cry02	1914	*cryIA(b)* gene (from *Bacillus thuringiensis*)		[69]
		Cry03/ Cry04	211	Crossborder sequence: CDPK-promoter/*cryIA(b)* gene		
	Control	Ivr1-F/ Ivr1-R	226	exon 3 of *ivr1* gene: (corn-specific single copy gene)		
MaisGard MON810 maize (Monsanto, USA)	ID (nested PCR)	mg1/mg2	401	Crossborder sequence: CaMV 35S promoter/ intron 1 of *hsp70*	[70]	[72]
		mg3/mg4	149	Crossborder sequence: CaMV 35S promoter/exon 1 of *hsp70*		
	Control (nested PCR)	hm1/hm2	175	*HMG* gene (highly conserved sequence)	[71]	
		hm3/hm4	149			
Bt-11 (Novartis Seeds, USA)	ID	IV01/ CR01	431	Crossborder sequence: intron 6 of *adh1*-1S gene/*cryIA(b)* gene	*cryIA(b)* [73], *pat* [74], CaMV [75], CDPK [76], PEPC [77], *hsp70* [78], *adh1*-S1 [79]	[80]
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)		PE01/ CR01	619	Crossborder sequence: PEPC promoter/*cryIA(b)* gene		
MaisGard MON810 maize (Monsanto, USA)		HS01/ CR01	194	Crossborder sequence: *hsp70* intron 1/*cryIA(b)* gene		
LIBERTY T14 or T25 (Hoechst Schering AgrEvo, Germany)		GM03/ PA01	231	Crossborder sequence: CaMV 35S promoter/*pat* gene		
Bt-11-,Event 176-, MON810-, LIBERTY-maize	Scr	CM01/02	220	CaMV 35S promoter		
	Control	ZE01/02	329	*ze1* gene (corn-specific single-copy gene)		
Maximizer maize (Plant Genetic Systems, Ciba Seeds)	ID (nested PCR)	CRY1A1/ CRY1A2	420	*cryIA(b)* gene from *Bacillus thuringiensis)*	[73]	[82]
		CRY1A3/ CRY1A4	189			
	Control (nested PCR)	ZEIN1/ ZEIN2	485	*ze1* gene (corn-specific single-copy gene)	[81]	
		ZEIN3/ ZEIN4	277			

Table 3 (continued)

GMO product (company)	Scr/ID	Primer	Amplicon length (bp)	Target gene or genetic element	Target sequence reference	Reference
GM maize (no further specification)	ID	Forward/ reverse	540	Crossborder sequence: CaMV 35S promoter/*dhfr* gene	[83]	[12]
		Forward/ reverse	840			
SeedLink maize (Plant Genetic Systems)	ID	Forward/ reverse	160	*barnase* gene	[84]	
		Forward/ reverse	235	*barstar* gene		
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)	ID	btsyn f1/ btsyn r1	151	*cryIA(b)* gene (from *Bacillus thuringiensis*)	[60]	
GM maize (Hoechst Schering AgrEvo, Germany)		bar-af1/ bar-ar	278	*bar/pat* genes from *Streptomyces viridochromogenes s. hygroscopicus*		
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)		CRYFZ 1/ CRYFZ 2	150	*cryIA(b)* gene (from *Bacillus thuringiensis)*		

i.e. DNA from plant viruses and bacteria, because of the risk of false positives. Therefore, a sample with a positive signal in *P-35S/nos3′* screening should be analysed for naturally occurring CaMV and *A. tumefaciens* infection, respectively [64]. However, it should be considered that the host range of the CaMV is restricted to cruciferous plants such as oilseed rape, and that the *nos3′* terminator sequence is found only in certain strains of *A. tumefaciens*, which are pathogenic to certain crop species. As to the *A. tumefaciens* frequently found in soil, it is generally not virulent, i.e. it does not carry the Ti-plasmid with the T-DNA and the oncogenes. Thus, the *nos3′* gene and its control elements are not present in these naturally occurring strains.

Most of the methods so far available concerning GMO screening and identification are listed in Table 3, Table 4, and Table 5. Where available, the reference where the original target sequence was published is also listed. Whereas in the past the focus was on development of screening methods, recently more emphasis has been given to the development of identification methods. *P-35S*, *nos3′* and *npt*II were widely used as GMO markers for screening purposes, whereas for identification purposes mostly cross-border sequences were reported.

Another strategy for GMO identification recently discussed makes use of amplified fragment length polymorphism (AFLP), a DNA fingerprinting method, which has already been used successfully to discriminate between and identify plant varieties [109, 110], including processed agricultural materials [111]. Interestingly, AFLP has been investigated for its potential in the combined identification of variety genotypes and the monitoring of very low levels of GM materials. Recent experimental findings indicated that the AFLP technology could be adapted for the detection of genetic modifications by using a GMO-specific primer in conjunction with a primer specific for the surrounding genomic region [60, 112].

Windels and co-workers [112] presented an anchored PCR strategy for the development of a line-specific GMO detection procedure. Subjecting the target DNA to restriction digestion and subsequent ligation of adapter oligonucleotides to the end of each restriction fragment, adapter primers were designed specific to the adapter sequence. GMO-specific anchor-primers were designed according to the *P-35S* and *nos3′* GMO marker sequences. With a combination of these primers it was possible (a) to amplify cross-border (or junction) fragments between insert and plant DNA, (b) to characterise, by subsequent sequencing, the line-specific locus of transgenic integration and (c) to design primers specific for the identified plant DNA sequences [112]. Although the AFLP approach is highly informative, some of the drawbacks of this technique include the use of multiple procedures in the protocol, high costs, the use of radioactivity and laboriousness.

Only a continuous survey of all data available on GMOs – especially the introduced genetic elements and their integration sites, not only for GM products approved for market release but also for any other GMO released for field trials worldwide – can guarantee a comprehensive monitoring of GMOs. Within the EU, preliminary efforts towards the establishment of a database providing at least part of the relevant information have already been made [60]. These efforts will be continued and extended within the European Commission's Joint Research Centre project "Development of a GMO register consisting of a database and accompanying bioinformatic tools designed for monitoring purposes as required under Directive 2001/18/EEC" [113].

Table 4 PCR methods in GMO detection for soybean. *RR* Roundup Ready

GMO product (company)	Scr/ID	Primer	Amplicon length (bp)	Target gene or genetic element	Target sequence reference	Reference
RR soybean (Monsanto, USA) dried soybean, soybean flour, extruded defatted soya acid, alcohol-precipitated soya concentrate	Scr	35S-1/-2	195	CaMV 35S promoter		[47][]
	ID (multiplex PCR)	SPA/SPB	320	Crossborder sequence: CaMV 35S promoter/ *EPSPS* gene		
	Control	LE1/LE2	407	*le1* gene (soya-specific single copy gene)	[85]	
RR soya lecithin (Monsanto, USA)	ID (multiplex PCR)	SPA/SPC	120	Crossborder sequence: CaMV 35S promoter/ *CTP* sequence		
	Control	LE5/LE6	180	*le1* gene (soya-specific single copy gene)	[85]	
RR soybean (Monsanto, USA)	Scr	35S-1/-2	195	CaMV 35S promoter	[86]	[27]
		NOS-1/ NOS-2	180	*nos* terminator (from *Agrobacterium tumefaciens*)		
	Control	plant-1/-2	500–600	Non-coding region from chloroplast genome	[87]	
Raw and fractionised RR soya lecithins (Monsanto, USA)	ID	p35s-af2/ petu-ar1	172	Crossborder sequence: CaMV 35S promoter/ *CTP* sequence		[44]
	Control	sole-af1/2	145	*le1* gene (soya-specific single copy gene)	[85]	
RR soybean (Monsanto, USA)	ID	p35s-f2/ petu-r1	172	Crossborder sequence: CaMV 35S promoter/ *CTP* sequence	[88]	[89]
Glyphosate-tolerant soybean seeds, Code No. 9396 (Pioneer Hi-Bred International, Inc. Iowa, USA)	ID (nested PCR)	GM05/	447	Crossborder sequence: CaMV 35S promoter/ *EPSPS* gene	[88]	[29]
		GM07/ GM08	169	Crossborder sequence: CaMV 35S promoter/ *CTP* sequence		
	Control (nested PCR)	GM01/02	414	*le1* gene (soya-specific single copy gene)		
		GM03/04	118			
Glycine max soybean (non-GMO) meat products	ID	GM01/ GM02	414	*le1* gene (soya-specific single copy gene)	[85]	[90]
		GM03/04	118			
	Control	TR03/ TR04	137	18S-rDNA (highly conserved sequence)	[68]	
RR soybean (Monsanto, USA)	ID (nested PCR)	RR01/ RR02	509	Crossborder sequence: *CP4 EPSPS* gene/ CaMV 35S promoter		[38]
		RR05/ RR04	180	Crossborder sequence: *CP4 EPSPS* gene/ *CTP* sequence		
	Control (nested PCR)	GM01/02	414	*le1* gene (soya-specific single copy gene		
		GM03/04	118			
		TR03/04	137	18S-rDNA (highly conserved sequence)		
RR soybean (Monsanto, USA)	ID	Forward/ reverse	475	*nptII* gene	[91]	[12]
RR soybean (Monsanto, USA)	ID	CAM/CTP	110	Crossborder sequence: CaMV 35S promoter/ *CTP* sequence		[60]
		EPS 1/ NOS a	147	Crossborder sequence: *CP4 EPSPS* gene/*nos* terminator		
	Control	LEC 1/ LEC 2	164	*le1* gene (soya-specific single copy gene)		

Table 5 PCR methods in GMO detection for tomato, potato, sugarbeet, cotton, papaya, alfalfa and tobacco

GMO product (company)	Scr/ID	Primer	Amplicon length (bp)	Target gene or genetic element	Target sequence reference	Reference
Flavr Savr tomato (Calgene., USA), Changin potato (Station fédérale de recherche en production végétale de Changin, Switzerland), glyphosinate-resistent sugarbeet (AgrEvo, Germany)	Scr	35S-1/35S-2	195+390 (in tomato)	CaMV 35S promoter	[86]	[27]
		Tn5-1/Tn5-2	173	*nptII* gene (from transposon Tn5)	[92]	
	Control	plant-1/-2	500–600	Non-coding region from chloroplast genome	[87]	
Changin potato (Station fédérale de recherche en production végétale de Changin, Switzerland), B33-Invertase potato (IGF, Germany)	Scr	NOS-1/NOS-2	180	*nos* terminator (from *Agrobacterium tumefaciens*)	[86]	
	Control	plant-1/-2	500–600	Non-coding region from chloroplast genome	[87]	
Flavr Savr tomato (Calgene, USA)	ID	PCR1/FS01	427	Crossborder sequence: CaMV 35S promoter/ *PG* gene	[93, 94]	[58]
	Scr	Tn5-1/-2	173	*nptII* gene (from transposon Tn5 on pBIN19 from *A. tumefaciens*)	[95]	
	Control	TR03/04	137	18S-rDNA (highly conserved sequence)	[68]	
GM Desiree potato, GM Rustica potato (*gbss*-antisense constructs)	Scr (nested PCR)	Tn5-1/-2	173	*nptII* gene	T-DNA [96], *gbss* [97, 98], B33 [99]	[100]
		T-ocd-1/ T-nos-2	432	T-DNA (from modified pBIN19 / near right border)		
		T-gene-III-1/ T-lacI-2	405	T-DNA (from modified pBIN19 / near right border)		
		T-lacZ-1/ T-M13-2	409	T-DNA (from modified pBIN19 / near left border)		
	ID (nested PCR)	T-geneIII-1/ T-M13-2	3800	Crossborder sequence: T-DNA/B33/link/gbss-AS/ T-DNA		
		B33-1/ gbss-as-2	580 (instead of 530)	Crossborder sequence: B33/link/gbss-AS (incl. confidential adapter seq.)		
	Control	universal -1/-2	550	Non-coding region from chloroplast genome	[87]	
GM tomato (Zeneca)	ID	Forward/reverse	472	Antisense *PG*/ CaMV 35S promoter	[101]	[12]
		Forward/reverse	478	Sense *PG*/CaMV 35S promoter		
		Forward/reverse	943	*nptII/nos* promoter		
		Forward/reverse	658	gene *IIIA/nos* terminator		
		Forward/reverse	890	CaMV 35S promoter/*nos* terminator		
		Forward/reverse	401	*nptII* gene/*ocd* gene		
		Forward/reverse	660	*ocd* gene/gene *IIIA*		
		Forward/reverse	180 (380)	*PG* gene (polygalacturonase)		
GM potato (no further specification)	ID	Forward/reverse	502	CaMV 35S promoter/ *PVX cp* gene	[102]	
GM cotton (DuPont, no further specification)	ID	Forward/reverse	642	*als* gene	[103]	
GM papaya (no further specification)	ID	Forward/reverse	674	*gus* gene	[104]	
GM alfalfa (no further specification)	ID	Forward/reverse	1097	*gus* gene	[105]	
		Forward/reverse	785	*nptII* gene		
GM tobacco (no further specification)	ID	Forward/reverse	880	P-TA29 promoter	[106]	
GM potato (B33-INV, no further specification)	ID	B1/B2	839	*aphIV* gene (hygromycinphosphotransferase)		[39]
	Control	A1/A2	550	Chloroplast tRNA gene		
Nema 282F tomato (Zeneca)	Control	PG34L/PG34R	383	*PG* gene (sense)	[107, 108]	[41]
	Scr		180	*PG* gene (antisense-construct)		
	ID	PG34L/B1	351	Crossborder sequence: *pg* gene/*nos* terminator		

PCR methods for GMO quantification

A major drawback of conventional PCR is the lack of accurate quantitative information due to the influence of the amplification efficiency (E). If the reaction efficiency for each amplification cycle remained constant, the concentration of DNA following PCR would be directly proportional to the amount of initial DNA target. Unfortunately, E is not a constant parameter but varies between different reactions, as well as within one reaction, particularly in the later cycles of the PCR, when products are formed at an unknown reaction rate and in a non-exponential fashion. In order to have maximum sensitivity, product formation is indeed measured when the amplification reaches the maximum product yield (known as the "plateau phase"), i.e. when the correlation between the product concentration and the number of the initial target molecules is very poor [9, 11, 114, 115, 116]. Thus, conventional PCR relies on end-point measurements, when often the reaction has gone beyond the exponential phase because of limiting reagents. Other PCR-based techniques, such as quantitative competitive PCR (QC-PCR) and real-time PCR, have recently been developed, which address the problems of establishing a relationship between the concentration of target DNA and the amount of PCR product generated by the amplification (for a detailed discussion of quantitative PCR approaches, please see [117]).

For relative GMO concentrations in food mixtures, the quantification of a GM marker has to be normalised to a plant-specific reference gene [118, 119, 120]. 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 the plant reference gene. With the assumption that the GMO material has been submitted to the same treatment as the non-GMO material, the measurement can be expressed as a genome/genome (g/g) or weight/weight (w/w) percentage.

Concerning the quantification of a GMO-specific gene in a mixture, such as in quantitative GMO analysis, there is currently an intense debate over how the GMO concentration should be expressed. At present, the argument is somewhat academic, since control laboratories first measure the DNA concentration in the sample and then for equal amounts of DNA they measure the number of copies that are found for a GMO-specific sequence and for a crop-specific sequence. The ratio of both – taking into consideration the respective number of copies per genome – gives the percentage of the GMOs present. Opponents of this approach argue that it is very difficult to quantify DNA. In addition, the assumption that the g/g ratio is equal to the DNA w/w ratio is probably an oversimplification since the genome size of crops is not a constant value. Variations of up to 25% within individual plant varieties have been reported [121, 122]. Therefore, new methods have been developed where the quantification of the reference gene and the GM marker gene are performed in the same reaction mixture (multiplex PCR). This approach allows the determination of the GM genome-copy/genome-copy ratio without the use of the DNA weight/concentration in the calculation. This ratio can be considered to be equal to the GM cell/cell ratio, generally equal to the GM w/w ratio [123]. However, there is currently no real valid proposal to substitute the g/g ratio with some other, more manageable units of measurement.

By reducing the influence of the varying amplification efficiency, the accuracy of the quantitative information obtained by PCR can be improved. The analytical strategies can be divided into two groups:

1. Co-amplification of target analyte with an internal standard, which allows a correction for the decrease in reaction efficiency, such as in QC-PCR and in double QC-PCR.
2. Measurement of the PCR amplicon in an early stage of the reaction, when the efficiency is still constant and therefore the product concentration still well correlates with the concentration of the initial target molecules, such as in PCR-ELISA and real-time PCR.

QC-PCR involves co-amplification of unknown amounts of a specific gene target and of known amounts of an internal control template in the same reaction tube by the same primer pair. A small difference between target and control sequence (<40 bp.) makes it possible to distinguish between the two reaction products. Each sample is amplified with increasing amounts of competitor, while keeping the sample volume/concentration constant. Quantification is achieved by comparing the equivalence point at which the amplicon from the competitor gives the same signal intensity as the target DNA on stained agarose gels [124]. The procedure assumes that the amplification reactions of the target sequence and the internal standard (i.e. the competitor) proceed with the same efficiency in any phase of the reaction, including the plateau phase.

The QC-PCR method described by Studer and co-workers [125] for the quantification of Roundup Ready (RR) soybean and Maximizer maize was successfully tested in an inter-laboratory trial with 12 European control laboratories [126]. Hardegger and co-workers [127] developed a QC-PCR screening method based on the quantification of the *P-35S* promoter and *nos3′* terminator. However, the fact that the copy number of these generic markers may vary between different GM lines needs to be considered when this method is applied.

Wurz and co-workers [118] reported findings of the assessment of the so-called double QC-PCR, a technique in which the concentration of soybean DNA in different samples is first normalized using a QC-PCR quantification of the soybean-specific lectin *le1* gene. When the same samples are submitted to a second QC-PCR for a GM marker, relative quantification can be established. However, since the generation of calibration curves is rather complex and the accuracy depends on various factors, Wurz and co-workers [118] only used one competi-

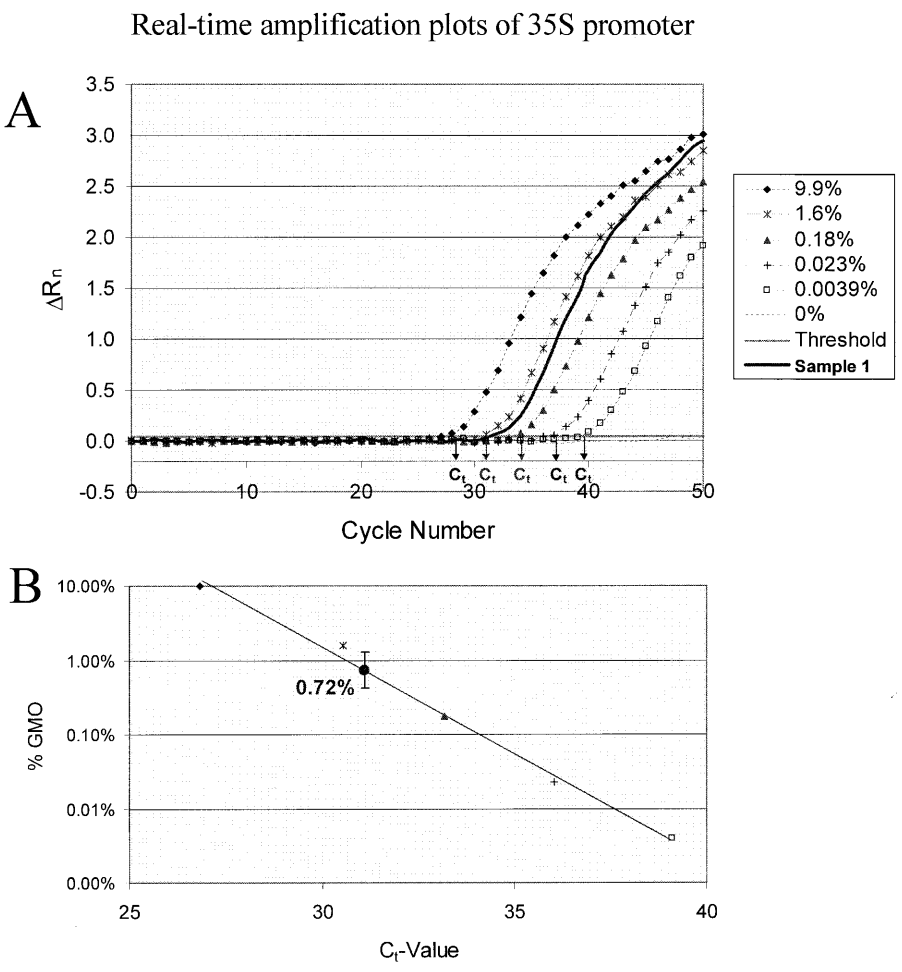

Fig. 2a,b Real time polymerase chain reaction (PCR). **a** Diagram showing the accumulation of the target analyte *P-35S* at six different ratios of genetically modified organism (GMO)/non-GMO material (w/w). PCR product formation is visualised in real time by taking fluorescence measurements (ΔR_n) at each cycle. The initial template concentration is determined on the basis of the threshold cycle(C_t) i.e. the PCR cycle at which fluorescenceis first detected to be statistically significant above background. C_t is inversely proportional to the log of the number of target copies present in the sample. **b** Linear regression diagram showing the logarithmic relationship between the GMO/non-GMO ratios and the C_t values

tor concentration, equivalent to 1% GM soybean (RR). Therefore, the method could only discriminate if a sample contained more or less GM material compared to the calibration concentration of 1%. Within this determination, some degree of uncertainty could not be avoided. More recently, Hupfer and co-workers [120] described a double QC-PCR method for the quantification of Bt-maize, in which multiple competitor concentrations were used for the quantification of the amount of transgenic DNA, as well as for the quantification of the total amount of amplifiable maize DNA. This allowed a good correlation between the actual and measured GMO concentration, even when the amount of amplifiable DNA was reduced by a heat treatment to less then 20% of the initial amount. Furthermore, they showed that the reduction of amplifiable DNA could not be observed by UV measurement, which demonstrates the need for an accurate quantification of both GM and endogenous plant marker. The use of double QC PCR might reduce the inter-laboratory differences observed in ring trial studies.

PCR-ELISA uses the strategy of the second group and can be quantitative when the PCR is stopped before a significant decrease in amplification efficiency occurs. ELISA has been used to quantify the relatively low amounts of PCR products [128, 129]. Despite the fact that relative quantification using PCR-ELISA has been applied in different fields [130] and that a GMO detection kit using PCR-ELISA has been commercialised (D-Genos, Angers, France), this technique has not been widely adopted for accurate GMO quantification purposes.

Another strategy of the second group that improves the accuracy, specificity and throughput of quantitative PCR is “real-time PCR” [131]. This technique was originally developed in 1992 by Higuchi and co-workers [132] and is rapidly gaining popularity due to the introduction of several complete real-time PCR instruments and easy-to-use PCR assays. A unique feature of this PCR technique is that the amplification of the target DNA sequence can be followed during the whole reaction by indirect monitoring of the product formation. Therefore, the conventional PCR reaction has to be adapted in order to generate a constant measurable signal, whose intensity is directly related to the amount of product formed. Real-time detection strategies rely on continuous measurements of the increments in the fluorescence generated during the PCR. The number of PCR cycles necessary to generate a signal statistically significant above the noise is taken as a quantitative measure and is called cycle threshold (C_t). (Fig. 2a) As long as the C_t value is measured at the stage of the PCR where

Table 6 Chemistry and instruments for specific and non-specific real-time PCR

Non-Specific	Specific
SYBR Green I (molecular probes) [139], Ethidium bromide [132]	Taqman [118, 123133, 137] FRET [139] Molecular beacons [140, 142, 143, 144] Scorpions [141]
ABI 5700 (Applied Biosystems) [146]	ABI 7700, ABI 7900 HT (Applied Biosystems) [118, 133, 145] LightCycler (Roche Molecular Biosystems) [147] Smart Cycler (Cepheid) [148] iCycler (Bio-Rad) [149] Mx4000 (Stratagene) [150]

the efficiency is still constant, the C_t value is inversely proportional to the log of the initial amount of target molecules (Fig. 2b).

Currently, various techniques are available for indirect monitoring of the PCR. For the use of real-time PCR in GMO detection it is important to distinguish between specific and non-specific PCR monitoring. Specific PCR monitoring, such as in the TaqMan assay, might reduce or eliminate the need for confirmation/validation testing, since non-specific amplification is not monitored. Furthermore, it might allow the simultaneous monitoring of multiple specific PCRs (the transgene and an endogenous reference gene) in the same reaction tube (multiplex PCR). When non-specific PCR monitoring is used, such as with the use of SYBR Green I, confirmation/validation testing is still necessary to confirm that the monitored signal came from the target sequence. In addition to the confirmation/verification methods described before, real-time PCR allows the determination of the melting temperature of the reaction product, by the use of melting curves. However, the reliability of this method will not exceed the reliability of size verification on an agarose gel. Obviously, simultaneous monitoring of different reactions is not possible.

The specificity of a real-time PCR method depends on (1) the chemistry which is used to allow monitoring of the amplification reaction and, (2) the instrument used to monitor the signal. Table 6 gives an overview of the currently available chemistries and instruments for real-time PCR categorised as non-specific and specific.

Wurz and co-workers [118] described how real-time PCR is amenable to relative quantification of GM soybean. Two different quantification reactions were applied to calculate the w/w percentage of GM soybean as a fraction of the total amount of soybean in a sample: a first one for the absolute quantification of total soybean DNA and a second for the absolute quantification of GM soybean DNA. Va and co-workers [133] described a similar method for the quantification of Maximizer maize and RR soybean and demonstrated the suitability of real-time PCR for the relative quantification of GM material in different food ingredients. The authors also reported how the combined quantification of the GM marker and an endogenous reference gene could be made in a single tube by using a multiplex PCR. Therefore, the quantitative PCR analysis of each sample was not affected by random differences in experimental factors such as pipetting errors, while the internal standard is an improved control of false negative results. The use of multiplex PCR in the quantification is made possible by the use of different reporter dyes, which can be detected separately in one reaction tube. Multiplex reactions are not only an economical way of doing PCR, they also allow accurate relative quantification without previous estimation of DNA quantity or copy numbers. With a multiplex reaction, a direct relationship between percentage GMO and the results of the real-time PCR can be established. This reduces the variation and permits accurate data interpretation by simple statistical evaluation of the quantification results [134]. Due to the above-mentioned advantages, multiplex real-time PCR is increasingly applied in genetic analysis [135, 136]. Siler and co-workers [137] developed an assay similar to that described by Va and co-workers [133], with an additional assay for the *P-35S* promoter and the *nos3′* terminator. Höhne and co-workers [138] successfully applied to GM maize a multiplex real-time PCR assay that employed *zein* as the endogenous reference gene and the *P-35S* promoter as a GMO marker of four different types of GM corn. A detection limit of 0.01% GM corn/non-GM corn was obtained. Pijnenburg [123] developed a multiplex assay for cured and processed tobacco leaf, a matrix rich in PCR-interfering compounds.

Besides the possibility of accurate quantification, the advantage of real-time PCR is the ability to increase the sample throughput compared to other quantification techniques, as post-PCR analysis is reduced to data treatment. Furthermore, with real-time PCR, the possibility of introducing variability and false positives is reduced. Since both amplification and detection are combined in one step carried out in a closed tube, the risk of cross contamination with PCR amplification products is minimised [133].

Currently, real-time PCR can be considered the most powerful tool for the detection and quantification of GMOs in a wide variety of agricultural and food products. While multiplex PCR formats with an endogenous reference gene will be able to increase accuracy, precision and throughput of this technique, a more comprehensive evaluation of real-time protocols and formats

(including data treatment) needs to be performed in the near future, possibly through inter-laboratory ring trials.

Validation of PCR methods

An increasing number of food control laboratories are adopting PCR as the technology of choice for GMO detection. The first official methods that have been validated in ring trials were published in the Swiss Food Manual and the German official collection of test methods in accordance with Article 35 of the German Food Act [39, 40, 41, 57]. However, international standardisation and validation of GMO analysis methods by harmonised and accepted protocols is still in its early phases. Standardisation bodies, such as CEN, and the French Standardisation Association (AFNOR Paris, France), have undertaken activities in this area and produced preliminary guidelines for sampling strategies and GMO detection methods, respectively [25, 151].

The objective of the validation of an analytical PCR method is to demonstrate that the successive procedures of sample extraction, preparation and analysis will yield acceptable accurate, precise and reproducible results for a given analyte in a specified matrix. The process of validation allows the independent use of methods and results, which are comparable among each other.

Depending upon the intended purpose of the analysis, i.e. qualitative (screening/identification) or quantitative analysis, different validation parameters have to be evaluated [115, 152, 153, 154, 155]. For the validation of a qualitative analytical test system specificity/selectivity, sensitivity [matrix effects/inhibition, limit of detection (LOD)], accuracy/precision [repeatability (RSD_r), intermediate precision, reproducibility (RSD_R)] and robustness have to be established. In addition to these parameters, limit of quantification (LOQ), accuracy/trueness and linearity/working range have to be evaluated for a quantitative analytical test system.

Specificity is defined as the probability of obtaining a negative result given that there is no analyte present [156]. It can be established by determining the percentage of correct classification of a non-analyte-containing sample as GMO negative, i.e. 100% minus the false-positive rate [24]. Recently, the specificity testing of a maize-specific real-time PCR system revealed that a signal was obtained not only with the target analyte (maize invertase gene) but also with DNA from rice and millet, even though of a 50-fold lesser intensity [26].

Sensitivity is defined as the probability of obtaining a positive result given that the analyte is present [156]. It can be established by determining the percentage of correct classification of an analyte-containing sample as GMO positive, i.e. 100% minus the false negative rate [24]. The exclusion of false negatives, e.g. an inhibition due to matrix effects, can be controlled by the co-amplification of an internal control – additionally, two controls should be checked routinely: a negative control as a test for contamination and a positive control run close to the LOD as a sensitivity test [157].

The LOD is determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can reliably be detected. The LOD can be defined as the concentration at which 95% of the experiments give a signal (i.e. 95% sensitivity) and may be experimentally determined by at least three series of dilution in DNA background where each dilution should be analysed in eight replicates [157]. The LOD can also be described as $LOD=(3.3\ \sigma):S$ where σ is the standard deviation of response (e.g. fluorescence signal) and S is the slope of the calibration curve (e.g. Fig. 2) [152, 153]. The LOQ is determined by analysis of known samples and establishes the minimum level at which the analyte can be quantified. It can be described as $LOQ=(10\ \sigma):S$ [152, 153].

However, recently Kay and Van den Eede [158] have discussed the variability of the proportions of GMOs to non-GMOs in replicate homogenised laboratory samples and its implications for the determination of the LOD: the number of haploid genome copies of GM maize in a 100 ng DNA sample with 0.1% GM maize content ranges from 25 to 48 with a 94.3% probability, i.e. from 0.068% to 0.131%. Therefore, in a dilution series the number of copies used to prepare subsequent dilutions would heavily influence the sampling error. A way to solve this dilemma could be (a) immediate dilutions from the primary laboratory sample, (b) a statistical relevant number of repetitions and/or (c) larger analytical samples [158].

Jankiewicz and co-workers [159] carried out a semi-quantitative study of sensitivity limits of the official German PCR method §35 LMBG 23.02.22–1 [40]. Two different approaches for the determination of the detection limit were compared: the theoretical LOD determined from serial dilutions of target DNA with background DNA, and the practical LOD determined by examining certified reference material and thereby taking into account the effect of the matrix during DNA isolation. Whereas the theoretical LOD was 0.005% GMO/non-GMO (w/w) corresponding to a calculated 30 copies of RR tolerant soybean haploid genome or to a calculated 9 copies of *Bacillus thuringiensis* resistant (Bt) maize haploid genome, the practical LOD proved to be significantly (20×) higher at 0.1% GMO/non-GMO (w/w) corresponding to a calculated 596 copies of RR soybean haploid genome or to a calculated 185 copies of Bt-maize haploid genome [159]. However, Jankiewicz and co-workers [159] did not test lower concentrations of certified reference materials nor did they report any repetition of their experiments.

The accuracy of an analytical system is determined by validation parameters related to precision, and to, in case of a quantitative analytical test, trueness [26, 152]. Precision is defined by determining the following three parameters: (a) the intra-laboratory variation checked by repetition of experiments over a short time (repeatability), (b) the intra-laboratory variation over a prolonged

Table 7 Ring trials for validation of GMO detection methods. *AACC* American Association of Cereal Chemists, *BAG* Bundesamt für Gesundheit, Switzerland, *BgVV* Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Germany, *DMIF-GEN* EU-Project "Development of Methods to Identify Foods Produced by Means of Genetic Engineering", *JRC* Joint Reseach Center, European Commission, *KLZ* Kantonales Labor Zürich, Switzerland, *RRS* Roundup Ready soybean, *Bt-176* Bt-176 maize, *MV* mean value± standard value, RSD_r repeatability, RSD_R reproducibility

Co-ordinator, date, reference	Type of Assay	Matrix (GMO)	Number of Laboratories[a]	Performance
Qualitative PCR detection methods for screening				
Official Swiss method / Brodmann et al., 1997 [37]	PCR/ screening	Soybean raw material and lecithin	6	~93% correct results for 35S 80 results (2 false positives, 4 false negatives) ~90% correct results for *nos* 80 results (8 false negatives)
JRC/Lipp et al., 1999 [158]	PCR/ screening	Maize and soybean meals	29	98.8% specificity for 35S in maize 93.5% sensitivity for 35S in maize 97.9% specificity for 35S in soybean 98.4% sensitivity for 35S in soybean 100% specificity for *nos* in soybean 96.7% sensitivity for *nos* in soybean
JRC/Lipp et al., 2001 [162]	PCR/screening	Processed foods with soybeans and/or maize	23	96.1% specificity for 35S 98.1% sensitivity for 35S 98.2% specificity for *nos* 97.9% sensitivity for *nos*
Qualitative PCR detection methods for specific identification				
Official Swiss method /Brodmann et al., 1997 [37]	PCR/ identification	Soybean raw material and lecithin (RRS)	6	~96% correct results for RRS 88 results (4 false negative)
Official German method	PCR/ identification	Raw potato (B33-INV)	No data	~98% correct results
/L 24.01–1, 1997 [39]				163 samples (3 false negative, 1 false positive)
Official German method	PCR/ identification	Soybean raw material (RRS)	25 (–3)	100% correct results
/L 23.01.22, 1998 [40]				110 samples
Official German method	PCR/ identification	Raw tomato (Nema 282F, Zeneca)	18	100% correct results
/L 25.03.01, 1999 [41]				90 samples
DMIF-GEN, 1999 [60]	PCR/ identification	Soybean meals (RRS)	16 (–4)	100% correct results 96 samples
DMIF-GEN, 1999 [60]	PCR/ identification	Maize flour (Bt-176)	18 (–3)	~97% correct results 75 samples (1 false negative, 1 false positive)
BgVV, 2000 [164]	PCR/ identification	Processed foods with soybean/maize (RRS, Bt-176)	15	~89% correct results 108 samples (11 false positive, 1 false negative)
Quantitative PCR detection methods				
KLZ/Hübner et al., 1999 [126]	QC-PCR/ semi-quantitative GMO-specific method	Soybean meals and processed foods (RRS and Bt-176)	12	For 0.5% GMO samples 9% relative standard error For 1.0% GMO samples 2% relative standard error F246 determinations (no false-positive, no false-negative) 0.81 contingency coefficient (chi-square-test, For RRS-specific compared with 35S-promoter-specific QC-PCR

Table 7 (continued)

Co-ordinator, date, reference	Type of Assay	Matrix (GMO)	Number of Laboratories[a]	Performance				
DMIF-GEN, 1999 [60]	QC-PCR/ semi-quantitative GMO-specific method	Maize meals (Bt-176)	19	For 0.1% GMO samples ~97% correct results For 0.5% GMO samples ~64% correct results For 1.0% GMO samples ~82% correct results For 2.0% GMO samples ~95% correct results				
DMIF-GEN, 1999 [60]	PCR-ELISA/ semi-quantitative screening method	Soybean meals	13 (–2)	For 0% GMO samples 91% correct results For 1% GMO samples 100% correct results for 2% GMO samples 100% correct results for 0.1% GMO samples ~29% correct results (0.1% GMO correctly classified after comparison with 0.5% control)				
JRC/Van den Eede et al., 2000 [163]	Double Competitive PCR/GMO-specific methods	Maize and soybean meals(RRS and Bt-176)	23 (–9) for maize, 23 (–10) for soybean	for 0/0.1/0.5/1/2/5% RRS 70–75% correct results For 0/0.1/0.5/1% Bt-176 73–79% correct results For 2% Bt-176 46% correct results For 5% Bt-176 23% correct results				
BgVV, 2000 [164]	Realtime-PCR/ GMO-specific method	Soybean meals (RRS)	14 for ABI 7700, 6 (–2) for ABI 5700, 12 (–5) for LightCycler	~23–28% variation coefficient for ABI 7700 ~46–82% variation coefficient for ABI 5700 ~21–41% variation coefficient for LightCycler For 0.1% GMO samples ~28–46% variation coefficient For 0.5% GMO samples ~25–82% variation coefficient For 1% GMO samples ~25–49% variation coefficient For 2% GMO samples ~21–48% variation coefficient For 5% GMO samples ~23–31% variation coefficient				
BAG/Pauli et al., 2001 [165]	QC-PCR/ semiquantitative 35S-screening and GMO-specific methods with visual inspection	Maize and soybean meals (RRS and Bt-176)	21 for soybean, 23 for maize	For 1.4/1.8/3.0% GMO ~90% correct results for 35S-screening For 0.7/1.0% GMO ~35% correct results for 35S-screening For 1.4/1.8/3.0% GMO ~92% correct results for GMO-specific For 0.7/1.0% GMO ~41% correct results for GMO-specific				
	QC-PCR/ semiquantitative 35S-screening and GMO-specific methods with image analysis		7 for 35S-screening, 8 for GMO-specific methods		35S-RRS	35S-Bt176	RRS	Bt-176
				For 0.7% GMO	1.49± 0.47 MV	0.86± 0.34 MV	1.25± 0.14 MV	0.86± 0.18 MV
				For 1.0% GMO	2.14± 0.48 MV	1.39± 0.62 MV	1.94± 0.39 MV	1.35± 0.49 MV
				For 1.4% GMO	2.30± 0.54 MV	1.56± 0.34 MV	1.99± 0.33 MV	1.74± 0.40 MV
				For 1.8% GMO	2.17± 0.86 MV	2.00± 0.25 MV	1.92± 0.27 MV	2.30± 0.53 MV
				For 3.0% GMO	3.24± 0.80 MV	2.98± 0.68 MV	4.10± 1.57 MV	3.35± 0.62 MV
	Realtime-PCR/ 35S-screening and GMO-specific methods	Soybean meals (RRS)	10		35S-RRS		RRS	
				For 0.7% GMO	0.87± 0.42 MV		0.86± 0.17 MV	
				For 1.0% GMO	1.66± 0.39 MV		1.65± 0.56 MV	
				For 1.4% GMO	1.62± 0.38 MV		1.78± 0.71 MV	
				For 1.8% GMO	1.79± 0.43 MV		1.80± 0.51 MV	
				For 3.0% GMO	4.02± 1.30 MV		3.79± 1.30 MV	

Table 7 (continued)

Co-ordinator, date, reference	Type of Assay	Matrix (GMO)	Number of Laboratories[a]	Performance
Quantitative non-PCR detection methods				
JRC/Lipp et al., 2000 [166]	Immunoassay/ qualitative and quantitative detection	Dried soybean powder (RRS)	38	For correct identification of <2% GMO (qualitative): Confidence: 99% For 2% GMO quantification: Accuracy: 95.1% mean/true value, –4.9% bias/true value Precision: 7.3% RSD_r, 9.3% RSD_R For 1% GMO quantification: Accuracy: 95.2% mean/true value, –4.8% bias/true value Precision: 9.2% RSD_r, 12.3% RSD_R For 0.5% GMO quantification: Accuracy: 88.1% mean/true value, –11.9% bias/true value Precision: 12.4% RSD_r, 16.6% RSD_R
AACC/Stave, 2001 [167]	ELISA	Ground maize kernels and maize meals (MON810)	40	Repeatability: 6.5–18.5% RSD_r Reproducibility: 13.8–23.5% Accuracy/non-GMO: 96.7–100% Accuracy/GMO: 113–125%

[a] The numbers in parentheses is the number of participants rejected from the analysis because either they did not follow the protocol, or they have been considered as outliers, or they had not submitted a (full) data sheet

time period, i.e. on different days with different staff and different equipment (intermediate precision) and (c) the inter-laboratory variation demonstrated in a ring trial (reproducibility). For a quantitative analytical testing the recommended data should also include standard deviation, relative standard deviation (coefficient of variation) for repeatability and reproducibility and the confidence interval. Trueness should be reported as the percentage recovery by the assay of a known added amount of analyte or as the difference between the mean and the accepted true value of the reference material together with the confidence intervals [26, 152].

Robustness defines the stability of the method and should be demonstrated with respect to deliberate variations in method parameters (work instructions, storage conditions and stability of analytical solutions) [152].

Recently, Waiblinger and co-workers [160] validated a real-time PCR system for the quantification of soybean meals in an in-house study. Performing the analysis of DNA dilution series in five repetitions the LOQ was 50 genome copies for the lectin system and 20 copies for the RR-specific system, and the LOD was 5 copies for both systems. The range of confidence intervals (determining the precision) was between 23% (56 copies), 8% (10,000 copies) and 13% (50,000 copies) for the lectin system, and 46% (6 copies), and 11% (100 copies) and 17% (2,500 copies) for the RR-specific system. The recovery rates (determining the trueness) were reported as 82%, 102%, 110% and 108% for 0.1%, 0.5%, 1% and 5% RR-soybean meal respectively [160].

The adoption of official or validated methods, as an analytical tool for GMO detection in raw plant materials or plant-derived foods, is in its initial stage. An overview of ring trials performed so far is given in Table 7.

For qualitative screening and identification the PCR method's substantial validation parameters, such as specificity and sensitivity, are only available for some of the ring trials [161, 162]. Due to a lack of available raw data and insufficient analysis in the publications the other ring trials can only be evaluated according to the percentage of correct results [37, 40, 41, 60, 164].

The two screening methods validated by the JRC [161, 162] were, like the official Swiss method [37], based on the detection of *P-35S* promoter and *nos* terminator by PCR. The participants of the ring trials were free to apply their method of choice for DNA extraction (suggested preparation according to CTAB or Wizard method) and they were requested to optimise the PCR conditions for their own equipment and to purchase their reagents locally. The primer sequences were the only parameter on which modifications were not allowed, thus serving as a pivotal point in these studies. The PCR products were separated by electrophoresis and examined by comparison to length standards expecting a 195 bp amplicon for the *P-35S* promoter and a 180 bp amplicon for the *nos* terminator. The identity of the amplicon was confirmed by restriction enzyme analysis. The performance in both ring trials was very promising with specificities (correctly classified as GMO negative) above 96% and sensitivities (correctly classified as GMO positive) above 93%.

Concerning the validation of the screening detection method on non-food crops, the Task Force Genetically Modified Tobacco: Detection Methods of CORESTA [9] recently initiated a collaborative study to evaluate the performance of a dry tobacco leaf protocol adapted from the one validated by JRC [63].

The three ring trials for the official German methods were based on the PCR methods for specific GMO identification in raw materials: potato [39], soybean [40] and tomato [41]. They include a description of procedures for DNA extraction (CTAB method), PCR analysis and a

confirmatory test. The PCR is performed with suitable primers specific for the GMO under study and the amplification capacity of the extracted DNA is checked by an internal control. The amplicons are then examined by agarose gel electrophoresis for their correct size. The identity of the amplicon is confirmed by a Southern blot. The performances of ring trials performed demonstrated reliability above 97% correct results.

Ring trials on quantitative PCR methods include four semi-quantitative QC-PCRs [60, 126, 165], one PCR-ELISA [60], one double competitive PCR [163] and two real-time PCRs [164, 165]. For the validation of quantitative detection methods, various statistical approaches were taken in order to evaluate the performance. Additionally, differences in the experimental design accompanied by a lack of available raw data do not permit a comparison of performance between these ring trials.

Another two ring trials validated quantitative non-PCR methods based on an immunoassay [166] and on a Cry1Ab-specific ELISA test [167]. For further details please see Validation of immunoassay methods and Table 7.

GMO detection with analytical precision involves the consideration of a number of critical parameters with respect to quality control. Any laboratory using such methods is expected to carefully validate every single step of the analytical process in order to produce comparable results. Further validation studies, especially on the quantitative PCR methods, must be performed.

Protein analysis methods

Immunoassay – principle and limits

The process of producing GM plants involves the introduction of transgenes that encode novel proteins. An overview on recombinant proteins expressed in plants is given by Steinkeliner and Korschhineck [168].

Immunological techniques have become indispensable tools in the physiological, biochemical and molecular disciplines of plant science. Their principal attraction resides in the high specificity of the immunological reaction, which allows accurate recognition of an antigenic substance even in the presence of contaminating antigens and interfering compounds. The methodology is now routinely used for the rapid purification, visualisation and quantification of proteins, polysaccharides and even small molecules (haptens) to which antibodies have been induced by conjugating the molecule to a large immunogenic carrier protein. An extensive overview on various immunological methods for assessing protein expression in plants is given by Dumbroff and co-workers [169]. The design and development of immunoassays for detection of proteins including those from novel foods is described in Brett and co-workers [170]. Moreover, in a recent review of GMO detection methods applied to tobacco, the Task Force Genetically Modified Tobacco – Detection Methods of CORESTA [9] has highlighted the advantages and drawbacks of immunoassay methods used for GMO detection.

One of the major drawbacks of immunochemical assays is that their accuracy and precision can be adversely affected in a complex matrix, such as those found in many processed agricultural and food products. The possible causes for interference from the matrix have been attributed to non-specific interaction with the antibody by proteins, surfactants (saponins), or phenolic compounds, antibody denaturing by fatty acids and the presence of endogenous phosphatases or enzyme inhibitors. Moreover, detection and measurement may be rendered difficult by low levels of expression of transgenic proteins, the degradation associated with thermal treatments or by a poor antibody affinity of the commercially available source of antibodies.

Nonetheless, several immunoassay-based methods have so far been developed, with an increasing number of ELISA systems with specificity for marker genes widely used in plant genetic modification, e.g. neomycin phosphotransferase II (*npt*II) [171, 172, 173]. This protein has been expressed in and purified from GM cottonseed, potato tubers and tomato. A recently modified ELISA method also based on the detection of *npt*II has been successfully applied to a number of independently transformed lines in nine plant species [174]. The enzyme 5-pyruvylshikimate-3-phosphate synthase (EPSPS) has been demonstrated to be expressed in RR soybeans [88] for which an appropriate immunoassay method has been developed [175]. Other proteins for which immunochemical analysis methods have been developed include Bt insecticide Cry1Ab and herbicide-tolerant phosphinotricin acetyltransferase (PAT) proteins. However, it must be stressed that differences can occur in the expression of the protein between crop varieties. This makes the quantification more challenging. In addition, there are numerous Cry proteins and one must bear in mind that EPSPS occurs naturally among all plant species.

Various commercial ELISA kits for a variety of applications are becoming available. Of interest are kits that are reported to detect specific proteins in food crops, such as Bt Cry1Ac, Cry1C, Cry3A, Cry2A, Cry9C, CP4 EPSPS, and PAT. ELISA methods offer a high degree of automation and a high throughput of samples. However, it must be stressed that the content of the newly exposed proteins is not evenly distributed in the whole plant. For instance, in maize the highest values were mostly observed in leaves and not in the grain.

Field variants of the ELISA, such as lateral flow strips or dipstick kits, have been recently developed. They offer a semi-quantitative test of considerable practical value for testing in the field with simple laboratory set-ups [176].

Validation of immunoassay methods

As more methods based on immunoassays are in development, there is a strong need for validation in order to make an appropriate comparison of efficiency.

General background information and a discussion of criteria for the validation and application of immunochemical methods to the analysis of proteins introduced into plants and food ingredients using biotechnology methods is given by Lipton and co-workers [177]. Future needs for detection of new or modified proteins in novel foods derived from GMOs are described in Stave [176].

Two ELISA kits have been validated so far by collaborative trial studies. The performance of a diagnostic ELISA kit to detect CP4 EPSPS was assessed [166] in a European ring study performed under coordination of the JRC, involving 38 laboratories from 13 member states and Switzerland, to detect and quantify GM RR soybean. In this validation study the ELISA assay gave an incorrect assignment of GM status in only 1% of samples in which the GMO was present at a level of 2% or greater. The immunoassay demonstrated a good repeatability with RSD_r=7%, a promising reproducibility with RSD_R= 10% and a detection limit of approximately 0.35% GMO.

Another recent collaborative study was successfully performed (including 40 laboratories in 20 countries) to detect Mon 810 by a specific ELISA test based on Cry1Ab. The quantitative range was between 0.15% and 2.0% with a reproducibility RSD_R figure of better than 23% [167].

Alternative techniques for GMO analysis (pattern investigation)

Chromatography

Where the composition of GMO ingredients, e.g. fatty acids or triglycerides is altered, conventional chemical methods based on chromatography can be applied for detection of differences in the chemical profile. This has been demonstrated with oils deriving from GM canola for which high performance liquid chromatography (HPLC) coupled with atmospheric pressure chemical ionisation mass spectrometry was applied to investigate the triglyceride patterns [178]. The spectral identification was based on the diacylglycerols fragments and on the protonated triglyceride molecular ions. Quantification was performed using a flame ionisation detector (FID). In comparing the triglyceride patterns, it could be observed that the oils of the GM canola varieties had an increased content of triacylglycerols, showing more oxidative stability for high stearic acid canola oil as well as for high lauric acid canola oil. This result is consistent with others obtained in previous oxidative stability studies on soybean and high oleic acid canola oils from new varieties by using HPLC-FID [179, 180]. In addition, the fatty acid compositions have been measured by using gas chromatography coupled with FID to support the HPLC results.

However, it must be stressed that this methodology is only applicable when significant changes occur in the composition of GM plants or derived products. Moreover, it is a qualitative detection method rather than a quantitative method. For example, small additions of GM canola oil with an altered triglyceride composition to conventional canola oil will most probably not be detected given the natural variation of ingredient patterns.

Near infrared spectroscopy

Certain genetic modifications may alter the fibre structure in plants, whereas no significant differences could be observed in the content of protein and oil (e.g. RR soybeans). These could be detected by near infrared spectroscopy (NIR) as reported by Hurburgh et al. [181]. Sample sets of RR and non-RR soybeans have been used to develop discriminate analysis calibrations for various models of near-infrared spectrometers. The results obtained by the three NIR instruments varied slightly, but were promising in all cases. However, the capacity of NIR to resolve small quantities of GMO varieties in non-GMO products is assumed to be low, as is true for the chromatographic methods.

Microfabricated devices and microchips for DNA analysis

One of the challenges that the GMO analyst will face in the near future is the rapid pace of development of GM plants that feature new and multiple genes and genetic control elements. For example, Hemmer [12] has already reported that some approved GM crops contain neither the *P-35S* promoter nor the *nos* terminator. While the establishment of "gene registers" and the use of advanced bio-information systems can help in obtaining prior knowledge of the possible types of genetic modifications, new technologies and instruments will be needed for the high throughput and low cost detection of an increasing variety of genes. New technologies resulting from the merger of chip-based micro-systems such as micro-arrays and micro-fluidic systems [182, 183, 184] appear to be a promising area for GMO analysis applications. Although several authors have reported on PCR-micro-systems of different complexities [185, 186, 187], few examples of microchip applications to GMO analysis have been described so far [188, 189].

One micro-technology that can be applied to both DNA and protein analysis is surface plasmon resonance (SPR) [190]. Minunni and co-workers [191] have proposed the use of biosensor technologies, including SPR for screening purposes in GMO analysis. They have highlighted several advantages using this technology including fast time responses, ease of use and low costs. These investigators obtained good preliminary results with an electrochemical biosensor featuring *P-35S* promoter and *nos* terminator oligonucleotide probes immobilised as capturing agent on a screen printed electrode. The probes recognised the complementary DNA sequences when exposed to the target analyte in solution,

with the system being much more sensitive to the *P-35S* than the *nos* target. The reference analytes were synthetic oligonucleotides and PCR-amplified DNA samples from RR soybean certified reference material (Fluka). Similar results were obtained when the same probes were immobilised on the surface of a SPR sensor (BIAcore) [192].

Conclusions

The introduction of agricultural commodities derived from biotechnology could have a profound impact on society and the economy in the coming decade, if the projected technological advances in plant genetic modification and genomics can be realised. As more and more GM traits enter the worldwide market, the monitoring of very low levels of GM materials and the identification of variety genotypes will be a pre-requisite for the determination of seed purity and for the verification of non-GMO status of agricultural crops throughout the supply and marketing chain.

Prospects for the effective monitoring of GM traits in the development, quality assurance and post-release phases of the GMO agriculture will be favoured by the availability of gene registers and dedicated information systems. The JRC is endeavouring to develop a molecular register that contains information on the specific genetic modifications and the appropriate identification methods. However, the use of gene registers must be accompanied by ready access to certified reference materials, which should be achieved through increased cooperation among all the stakeholders in GM crop development, from seed developers to processors and manufacturers.

Because of its unparalleled sensitivity and specificity, PCR, in its various formats, is currently the leading analytical technology employed in the qualitative and quantitative analysis of GMOs. On the other hand, immunoassays are becoming attractive tools for rapid field monitoring of the integrity of agricultural commodities in identity preservation systems, whereby non-specialised personnel can employ them in a cost-effective manner.

The integration of conventional and new molecular tools for plant variety development will give rise to an increasingly wide range of GMOs with multiple gene constructs, i.e. with multiple analytical targets in the same product. Although at present these new analytical issues and challenges cannot be addressed with the available technologies, far-reaching possibilities may be realised in the near future, for example by research on the miniaturisation of analytical devices, and the consequent introduction of microchips and micro-fluidic systems for genetic analysis.

While GMO testing techniques are continually improving with respect to accuracy, reliability and speed, it should be stressed that their performance is strongly affected by sampling strategies and processes. Sampling plans are to be thoroughly evaluated and defined for each new crop material and/or ingredient. It is therefore necessary to work towards the establishment of internationally accepted and harmonised sampling protocols such as those being developed by the Working Group "Genetically Modified Foodstuffs" in Technical Committee CEN/TC 275 of CEN.

Finally, considering the potential economic impact of GMO commingling in the supply and marketing chain, it appears to be of paramount importance that analytical determinations of GMO presence/absence in food and agricultural products be made by the use of internationally validated and approved methodologies and standards. The adoption of official or validated GMO testing methods is, however, in its initial stage, and concerns only qualitative or semi-quantitative detection of GM raw agricultural products. Validation and harmonisation of quantitative methods will be needed to address compliance with GMO thresholds, which take into account the possibility of inadvertent commingling of GM with conventional materials during agricultural production, shipping or processing.

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