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REVIEW

Methods for detection of GMOs in food and feed

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Abstract This paper reviews aspects relevant to detection and quantification of genetically modified (GM) material within the feed/food chain. The GM crop regulatory framework at the international level is evaluated with reference to traceability and labelling. Current analytical methods for the detection, identification, and quantification of transgenic DNA in food and feed are reviewed. These methods include quantitative real-time PCR, multiplex PCR, and multiplex real-time PCR. Particular attention is paid to methods able to identify multiple GM events in a single reaction and to the development of microdevices and microsensors, though they have not been fully validated for application.

Keywords GMO traceability · Regulation and legislation · PCR methods · Sensor and microsensor · Validation of methods

International regulations covering the deliberate release of genetically modified plants and the future use of genetically modified feed and food

The debate surrounding the use of genetically modified (GM) plants in agriculture and agro-industry continues apace, involving a combination of scientific, social, and political aspects. There is as yet little unanimity among governments and legislators regarding the foreseeable risks that GM crops may pose to human health and the environment. The International Service for the Acquisition of Agri-biotech Applications (ISAAA) estimates that in 2007, some 12 million farmers cultivated GM crops over more than 114 Mha spread across 23 countries, worth in excess of US $6.9 billion. The major GM crop species are canola, maize, cotton, and soybean, and cultivation of these is concentrated in the developed countries which dominate global trade in these commodities. However, interest in producing GM crops is now growing rapidly in the developing world [1]. In contrast, the trend within the European Union (EU) is negative, because since the initial release of GM crops in the early 1990s, their cultivation has been inhibited by government regulation intended to protect human/animal health and the environment [2–4]. The issue of liability and redress surrounding the national/international trading of GM products was widely debated in March 2006 during the COP-MOP3 meeting held in Curitiba [5, 6], and a prominent view was that the prime reason for the marginal global relevance to international trade of GM crops was that a mass of at times controversial and confusing legislation has created too much legal uncertainty.

The US Government has applied the principle of substantial equivalence [7] to direct its legislation in the area of GM safety. This holds that a GM product is not distinct in essence from a conventional one, and thus its release can be considered under existing legislation (specifically, the Plant Protection Act (PPA), the Federal Food, Drug and Cosmetic Act (FFDCA), the Federal Insecticide, Fungicide and Rodenticide ACT (FIFRA) and the Toxic Substances Control Act (TSCA)). The current system used to regulate the use of GM technology in the field was defined by the 1986 "Coordinated Framework for Regulation of Biotechnology" (United States Federal Register, 26 June 1986, 51

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FR 23302) and the 1989 framework of the National Research Council (NRC) [8–12]. The responsibility for the various aspects of GM risk assessment is delegated to the Food and Drug Administration (FDA), the Department of Agriculture (USDA), the Animal and Plant Health Inspection Service (APHIS), and the Environmental Protection Agency (EPA). In the EU, the system is an amalgam of regulations, directives, and amendments, which have developed out of various decisions taken by the European Commission (EC), the European Parliament (EP), the relevant Council of Ministers, and each of the Member States. The EU system takes a process-based rather than a product-based approach to evaluate the risk posed by the cultivation of GM crops and the consumption of GM food and feed. The two earliest directives were 90/219/EEC (amended by 98/81/EC in 1998), which covered containment, and 90/220/EEC, which was concerned with the deliberate release of all genetically modified organisms (GMOs, including GM crops) into the environment. Since 17 October 2002, these have been superseded by 2001/18/EC, which explicitly adopted the precautionary principle, elaborated in the Cartagena Protocol (Preservation of Biodiversity), and recently revisited in Curitiba [13]. This regulates both the deliberate release of GMOs for experimental purposes and for commercial use within the EU of products containing GM crops, as well as microorganisms. The presence of GM material in food, feed, and food products is governed by 1829/2003, which insists on a labelling standard for all products containing GM-based materials. Specifically, materials delivered either directly to the consumer or via a third party must be labeled if their production has involved the use of GM materials—even if the product itself contains no DNA or protein originating from a GMO (as is the case for highly refined products, such as oil or starch). Labelling is required where the content of any authorized GM ingredient exceeds 0.9% of the food or feed product; in this case, the term "genetically modified" must appear in the list of ingredients immediately following the relevant ingredient. Below this threshold, the presence of GM material is considered to be accidental or technically unavoidable, and the product can be sold without labelling. For non-authorized GM ingredients, the threshold is set at 0.5%, provided that the source GMO has been pre-evaluated, and that an appropriate detection method for its presence is available. For seed, the threshold is 0%, i.e., all GM seed must be labeled according to 2001/18/EC. Table 1 summarizes the state of relevant legislation in various countries, whereas Table 2 compares the regulatory systems of the EU and the USA. The GM components of a food or feed are considered by some legislation as contaminants [14], resulting in a considerable demand for analytical methods capable of detecting, identifying, and quantifying either the presence of GM DNA or GM protein, at the farm gate, the processor, and the retailer level.

A recent conference, the 1st Global Conference on GMO analysis (Como, Italy, June 2008) [15], provided a forum for identification and discussion of the still unresolved issues in the development of appropriate testing methods, including: (i) sampling strategies; (ii) extraction methods; (iii) reference materials; (iv) multiplexing; (v) new targets and challenges; (vi) quality assurance and accreditation; (vii) harmonisation needs; (vii) economic impact of testing. This review recapitulates the current state of the art in GMO testing in light of these unresolved issues.

Qualitative methods for GM detection

Most current detection methods rely either on the polymerase chain reaction (PCR) to amplify transgene sequence(s), or on immunological methods (primarily ELISA, the enzyme-linked immunosorbent assay) to bind to a transgene gene product(s). Although specific DNA sequences can be detected by hybridization, it is PCR in its various formats (qualitative PCR, end-point quantitative PCR, and quantitative real-time PCR) which has been generally accepted by the regulatory authorities [16]. All PCR assays require that a minimum number of target DNA sequences be present in the template, and that the sequence of the target DNA is known. The extraction and purification of DNA from the sample is a particularly critical step [17, 18]. Refinements in PCR technology have been such that it has become the only reliable method to hand able to detect the presence of a specific DNA sequence from samples containing little and/or poor quality DNA. For example, it is possible to test for a target DNA sequence in heavily aged or highly processed samples [19]. The EU FP5 project DNA-TRACK (www.dsa.unipr.it/foodhealth/) explored a number of methods for the extraction, purification, and amplification of DNA from materials containing transgenic ingredients (Table 3). The effect of the various mechanical, thermal, and chemical treatments employed in foodstuff manufacturing (which all act to degrade DNA) on the ability to amplify target DNA sequences is shown in Table 4. Methods for DNA extraction were compared with respect to DNA yield, the presence of inhibitors, the cost per sample, and the time required per extraction (Fig. 1). Considering these parameters together the method based on CTAB was the best. It has low cost with respect to commercial kits, since it uses products commonly found in molecular biology laboratory, it gives good DNA yield (from 160 μg/ml for chicory to 1,970 μg/ml for peanuts), the time required for DNA extraction from 13 samples is 2 h, while the method requiring the longest time is the PVP method. The PVP method and the Roche kit allowed the

Table 1 State of legislation for labelling of substantially equivalent products containing GMOs

Country	Type of labelling	Degree of enforcement	Threshold (%)
Argentina	Voluntary	No legislation	
Australia	Mandatory	Enforced labelling policy	1
Bolivia		Planning introduction	
Brazil	Mandatory	Partially/not enforced	1
Cameroon		Planning introduction	
Canada	Voluntary	Enforced labelling policy	5
Chile		Partially/not enforced	
China	Mandatory	Enforced labelling policy	No level
Colombia		Planning introduction	
Croatia		Partially/not enforced	
Ecuador		Partially/not enforced	
Egypt		Planning introduction	
El Salvador		Partially/not enforced	
Ethiopia		Planning introduction	
European Union	Mandatory	Enforced labelling policy	0.9
Georgia		Planning introduction	
Hong Kong	Voluntary	Enforced labelling policy	
India		Planning introduction	
Indonesia	Mandatory	Partially/not enforced	5
Israel		Planning introduction	
Ivory Coast		Planning introduction	
Jamaica		Planning introduction	
Japan	Mandatory	Enforced labelling policy	5
Malaysia		Planning introduction	
Mauritius		Partially/not enforced	
Namibia	Planning	Planning introduction	
New Zealand	Mandatory	Enforced labelling policy	1
Nigeria		Planning introduction	
Norway	Mandatory	Enforced labelling policy	
Paraguay		Planning introduction	
Peru		Planning introduction	
Philippines	Voluntary	Planning introduction	5
Russia	Mandatory	Enforced labelling policy	0.9
Saudi Arabia	Mandatory	Enforced labelling policy	1
Serbia		Partially/not enforced	
Singapore		Planning introduction	
South Africa	Voluntary	Enforced	
South Korea	Mandatory	Enforced labelling policy	3
Sri Lanka		Partially/not enforced	
Switzerland	Mandatory	Enforced labelling policy	
Taiwan	Mandatory	Enforced labelling policy	5
Thailand	Mandatory	Partially/not enforced	5
Uganda		Planning introduction	
Ukraine		Partially/not enforced	
UAE		Planning introduction	
USA	Voluntary	Enforced labelling policy	
Uruguay		Planning introduction	
Vietnam		Partially/not enforced	
Zambia		Planning introduction	

almost complete elimination of inhibitory substances but the DNA yield was really low. In conclusion this evaluation showed that the most suitable DNA extraction method depends both on the food matrix and on the researchers' needs.

The quantification of GMO content, as required by EC 2001/18, is described in Recommendation 2004/787/EC, which requires the ratio between copy number of the GM event DNA and an endogenous (species-specific) reference target, expressed in terms of haploid genome equivalents.

Table 2 Summary of national GM regulation in EU and USA

Country	Regulatory system	GM products approved	Labelling requirements
European Union	Directive 2001/18 on the deliberate release into the environment of GMOs entered into force on 17 October 2002. Harmonized procedures and criteria for the case-by-case evaluation of potential risks: mandatory prior notification by applicants, accompanied by full environmental risk assessment, detailed information on the GMO, its release conditions, interaction with the environment, monitoring, waste and contingency plans, labelling and packaging proposals. Complex approval procedure involving competent national authorities, the EC and Council of Ministers Regulation 1829/2003 on GM food and feed, replacing the GM part of Regulation 258/97, entered into force on 7 November 2003 and applied as of 18 April 2004: authorization procedure for market placement of GM food and feed, including food and feed produced from GMOs, irrespective of whether there is DNA or protein of GM origin in the final product. Approval procedure simplified. The European Food Safety Authority (EFSA) charged with carrying out scientific risk assessment Regulation 1830/2003 on traceability and labelling of GMOs, entered into force on 7 November 2003 and applied as of 18 April 2004: strengthened rules on mandatory traceability and labelling Regulation 1946/2003 set controls on trans-boundary movements and unintentional movements of GMOs between Member States and export to third countries Regulation (EC) 65/2004 of 14 January 2004 established a system for the development and assignment of unique identifiers for GMOs Commission Regulation (EC) 641/2004 of 6 April 2004 detailed rules for the implementation of Regulation (EC) 1829/2003 of the European Parliament and of the Council of Ministers, as regards application for the authorization of new GM food and feed, the notification of existing products, and adventitious or technically unavoidable presence of GM material which has been given a favorable risk evaluation	24 GMOs have been approved by 2007, and authorization for 50 is currently pending	Mandatory labelling for all GMOs and GM products, including food and feed produced from GMOs but no longer containing GM material, unless the presence of GM material is adventitious and below 0.9%. 0.5% threshold for adventitious presence of non-approved GMOs, which have been assessed as risk-free
USA	1986 Coordinated Framework for Regulation of Biotechnology based on the equivalence principle, existing laws were applied to ensure the safety of GM products: the Plant Protection Act (PPA), the Federal Food, Drug, and Cosmetic Act (FFDCA), the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and the Toxic	163 plants intended for food or feed completed all required reviews for planting, food, or feed use	Proposals on voluntary and mandatory labelling

Table 2 (continued)

Country	Regulatory system	GM products approved	Labelling requirements
	Substances Control Act (TSCA). Responsible agencies: FDA (food and feed safety); APHIS (environmental safety of GM crops) and EPA (development and release for GM plants with pest control properties) Food and Drug Administration (FDA) 1992 Statement of Policy: Foods Derived from New Plant Varieties encouraged developers to work cooperatively with the FDA under a practice of (non-mandatory) consultations to allow the FDA to obtain information necessary to assess safety before commercialization Food and Drug Administration 2001 Draft Pre-market Notice Concerning Bio-engineered Foods gave draft guidance for industry voluntary labelling, indicating whether foods have or have not been developed using bioengineering		

This ratio has to be established for each species and for each transgene. As the reference target must be quantitatively stable in all possible host genetic backgrounds, a single copy gene with only one allele per locus represents the preferred option. The major analytical problems inherent in these requirements have been widely explored [22]. PCR-based tests for the presence of GM material have been classified according to their level of specificity [23] into those which target: (i) widely used sequences such as P-35S (CaMV 35S promoter), T-35S (CaMV 35S terminator), T-Nos (terminator of the nopaline synthase gene), bla (β-lactamase), and nptII (neomycin phosphotransferase II);

Table 3 Methods utilised for DNA extraction and purification from foodstuffs containing maize and soy ingredients

Short name	Manufactured by	Main steps of the method		Sample		Cost
		Lysis	Purification	Fresh weight (mg)	Dry weight (mg)	€/sample
DNeasy	Qiagen	Detergents	Salts, silica membrane	100	20	2.82
Nucleon	Amersham	SDS	Resin, chloroform, isopropanol	100	20	2.38
NucleoSpin	Macherey Nagel	CTAB	Silica membrane	100	100	2.33
Roche	Roche	Not known	Isopropanol	200	40	5.34
PrepMan	Applied Biosystems	Not known	Not known	100	20	1.40
WizardGen	Promega	Detergents	Salts, isopropanol	40	40	1.11
WizardPlus	Promega	G-HCl, SDS, proteinase K	Resin	300	100	2.65
Wizard Magnetic	Promega	G-HCl, SDS, proteinase K	Magnetic particles	300	100	
CTAB	Pietsch et al. [20]	CTAB	Chloroform, isopropanol	300	100	0.07
PVP	Kim et al. [21]	SDS	PVP, isopropanol, chloroform/isoamyl alcohol	100	20	0.27
QIAamp DNA Stool	Qiagen	Not known	Silica membrane	300	100	ND
Nucleospin Food	Macherey Nagel	Not known	Silica resin	300	100	ND
NucleoSpin Plant	Macherey Nagel	Not known	Silica resin and detergents	300	100	ND
GeneClean kit	Q-Biogene	Detergents and particles	Microcolumn membrane	300	100	ND
GenElute Plant	Sigma Aldrich	Detergents	Microcolumn membrane	300	100	ND

ND not determined

Table 4 Amplificability of DNA extracted with the purification methods Wizard®, NucleoSpin Plant (NSP), Geneclean®

Samples	Wizard®							NSP							Geneclean®						
	Clor	Le	Ze	35S	NOS	CRY	GM	Clor	Le	Ze	35S	NOS	CRY	GM	Clor	Le	Ze	35S	NOS	CRY	GM
Soy Flour[a]	+	+		−	−		+	+	+		−	−		−	+	+		+	−		+
Soy proteins[c]	+	+		−	−		+	+	+		−	−		+	+	+		−	−		−
Soy milk [b]	+	+		−	−			+	+		−	−		−	+	+		−	−		−
Soy steaks[c]	+	+		−	−		+	+	+		−	−		+	+	+		−	−		−
Tofu[c]	−	+		−	−		−	+	−		−	−		−	+	+		−	−		−
Soy biscuits [b]	+	+		−	−		−	+	+		−	−		+	+	+		+	−		+
Soy Snack[c]	−	+		−	−		−	+	+		−	−		+	+	+		−	−		−
Soy sauce[c]	−	−		−	−		−	+	−		−	−		−	+	+		−	−		−
Polenta[a]	+		+	+		−		+		+	−		+		+		+	−		+	
Chips[c]	+		+	−		−		+		+	+	+	−		+		+	+		+	
Cornflakes[b]	−		+	−		−		+		+	−		+		+		−	−		−	
Cracker[b]	+		+	−		−		+		+	+		−		+		+	−		+	
Maize bread[b]	+		+	−		−		+		+	−		+		+		+	−		+	
Maize biscuits[b]	+		+	+		−		+		+	−		−		+		+	−		−	
Chocolate[c]	−	−		−	−		−	+	+		−	−		−	+	+		+	−		−
Starch[c]	−		+	+		−		−		+	−		−		+		−	−		+	
Margarine[c]	−	−		−	−		−	+	+		−	−		−	−	−		−	−		−

Qualitative PCR amplification were performed as reported in ref. [17]

+ positive amplification; − negative amplification; target sequences: *Clor* (chloroplastic gene), *Le* (lectin gene), *Ze* (zein gene), *35S* (35S promoter), *NOS* (terminator), *CRY* (*Bacillus thuringensis* cryIA gene), *GM* (*Agrobacterium* sp. CP4-EPSPS gene)

[a] Mechanical treatment

[b] Mechanical and thermal treatments

[c] Mechanical, thermal, and chemical treatments

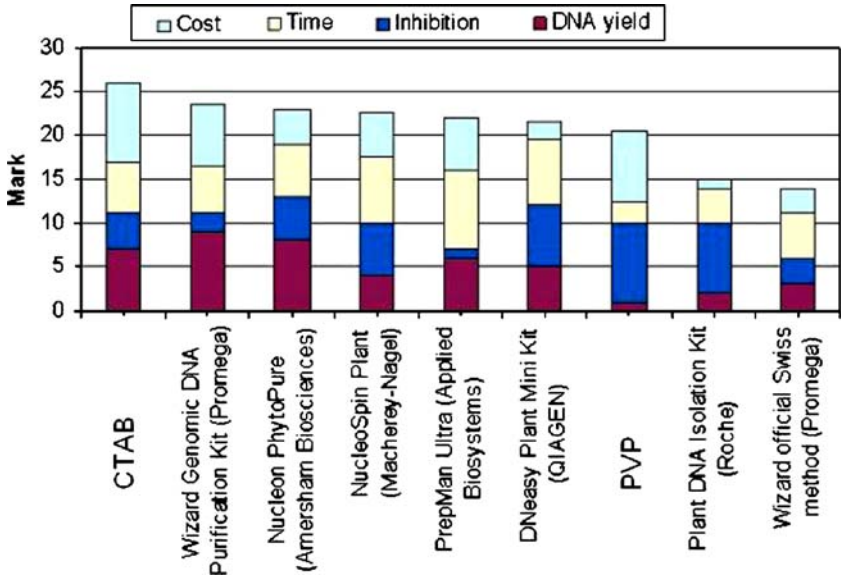

Fig. 1 Comparison of DNA extraction methods, considering yield of DNA, presence of inhibitors, cost per sample (based on manufacturers' bulk price), and the time needed to perform 13 independent extractions

(ii) sequences within a specific transgene; (iii) sequences which are construct-specific, an example being the junction between the promoter sequence used to drive the transgene and the transgene itself; and (iv) sequences which are event-specific, such as the transgene integration site (Fig. 2). A major limitation is access to transgene sequence information, although most of the common events are detailed at http://www.agbios.com/dbase.php. A number of suitable primer pairs have been developed over the last decade, but some of these have a rather limited application range. An increasing number of event-specific assays are now present in GM crops, for example, Roundup Ready (RR) in soybean [24–26], MON810 [27, 28], Bt11 [29], Starlink [30], NK603 [31], and MON863 [32] in maize, and Mon1445 and Mon531 [33] in cotton. Some methods suitable for the detection of "unknown events" have been presented [34], based on oligonucleotide arrays, rather than on PCR.

The outcome of PCR is not always completely unambiguous, so some post-PCR control is generally necessary to confirm sequence identity. Methods directed at this requirement have been extensively described elsewhere [34, 35], but are presented below in summary form:

- Checking amplicon size by gel electrophoresis. This can give a false positive where a non-target sequence of the same length as the target has been amplified. Where a restriction site(s) is present in the target sequence, verification can be improved by appropriate restriction digestion.
- Checking the amplicon sequence via a hybridization assay. Although this is a reliable method, it is also time-consuming and costly.
- Using nested PCR to discriminate between target and non-target amplicons.
- Sequencing the amplicon. This is the most reliable means of authentication, and where low cost DNA sequencing services are available, this is the preferred method.
- Exploiting peptide nucleic acid (PNA)-mediated PCR clamping. This procedure specifically and efficiently inhibits the amplification of sequences differing from

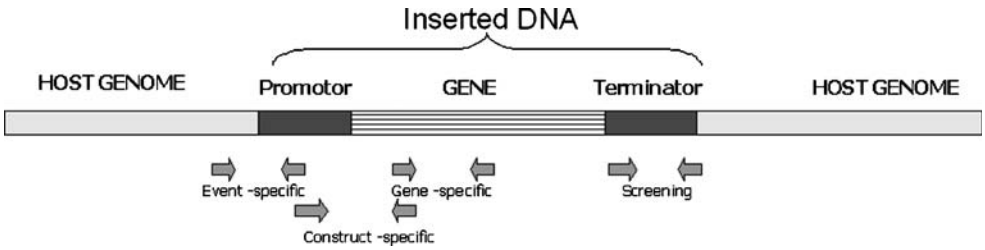

Fig. 2 Schematic of a typical transgene construct and four types of PCR-based assays. The host DNA is genomic DNA of the GM crop; the transgene consists of a promoter, the gene itself, and a terminator. *Arrows* indicate the primer pairs targeting particular sequences around and within the transgene integration site. Four assay types are illustrated, targeting: generic transgene sequences (P-35S, T-35S, T-Nos); sequences within the gene of interest; construct-specific junction sequences; and event-specific junction sequences

Table 5 Chemistry and instrument for specific and non-specific real-time PCR

	Chemistry	Instruments
Non-specific	SYBR green I	ABI 7700 (Applied Biosystems) [58]
	LC Green (Idaho Technology) [44]	HR-1 (high resolution melting instrument (Idaho Technology) [44]
Specific	TaqMan probe [45]	ABI 7700, ABI 7900 HT (Applied Biosystems) [58]
	MGB TaqMan probe [46]	
	FRET [47]	LightCycler (Roche Molecular Biosystems) [59]
	Molecular beacons [48–51]	Smart Cycler (Cepheid) [60]
	Plexor technology [52]	iCycler (Bio-Rad) [61]
	Scorpion [53]	Mx4000 (Stratagene) [62]
	LNA (locked nucleic acid) probe [54]	
	LUX (light upon extension) primers	
	Amplifluor [55]	
	FDSP (fluorophore double stranded probe) [56]	
	CPT (cycling probe technology) [57]	

the target by as little as a single base pair. It represents a rapid and effective means of assessing band identity, with some potential for semiquantitative assessment [36, 37].

Multiplex PCR methods

Since both the number of authorized transgenic events and the cultivation area of GM crops are rapidly increasing, there is a need to accelerate the methods for GM detection. One approach takes advantage of multiplex PCR, in which several primer pairs are included in the PCR to permit the simultaneous detection of multiple target sequences. Such systems have been developed for a number of construct-specific targets [38, 39]. Typically, the various PCR products are distinguished from one another on the basis of their differential migration through agarose gels, since this platform is both simple and cost effective. In a nonaplex (nine construct-specific primer pairs) PCR assay [40], eight GM maize varieties were successfully distinguished from one another in a sample containing 0.25% of each event. In this case, both agarose gel and capillary electrophoresis were used to detect the various amplicons, which ranged in size from 110 to 444 bp. The relatively large size of some of these PCR products implies that this assay would be unsuitable for highly processed products, in which the extent of DNA degradation generally precludes the survival of long stretches of intact DNA. The simultaneous detection of the transgenic events Bt11, GA21, MON810, and NK603 in maize has been achieved by exploiting transgene/plant genome flanking regions [41], with an LOD of 0.1% for each event. In this application, the various amplicons were distinguished from one another by labelling with different fluorochromes, so required the use of a DNA sequencing device. A particular advantage of this platform is that it can be readily adapted to a high-throughput mode.

Quantitative methods: uniplex and duplex real-time quantitative PCR

Quantitative real-time PCR (qRT-PCR) represents the most powerful current means of quantifying GM material in agricultural and food products [19]. It operates by continuously monitoring the amplification reaction, using the strength of the fluorescence signal to indicate the quantity of amplicon present [42]. The specificity of qRT-PCR depends on both the chemistry used to monitor amplification, and the instrumentation used to monitor the signal. Several detection chemistries have been developed (Table 5), the most widely used of which are TaqMan® and SYBR Green®. An overview of the currently available qRT-PCR instrumentation is also given in Table 5. When used to assess the quantity of GM material in a sample, two parallel reactions each containing the same amount of template DNA are commonly performed: one targets the endogenous reference sequence, and the other the GM-specific sequence. Quantification is achieved either by a comparison based on the cycle threshold of the two amplified sequences (the ΔCt method), or via titration against a standard curve. An alternative approach uses a multiplex reaction exploiting differential fluorochrome labelling for the two amplicons. There are some technical difficulties with this approach, centered around the overlapping of the emission spectra of the fluorochromes in common use. However, intercalating dyes (such as SYBR Green I and LC Green), which have a high affinity for double-stranded DNA and show enhanced fluorescence when bound to DNA, offer a useful alternative to fluorochromes. The monitoring of amplicon quantity is achieved in this case by applying a

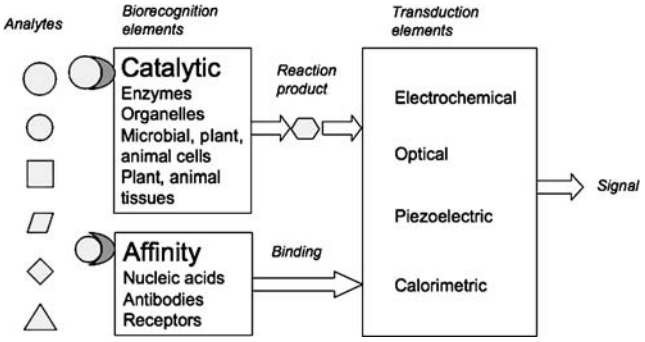

Fig. 3 Scheme illustrating the design of a biosensor. Analytes are recognized by a bioelement (e.g., enzymes, organelles, cells, tissues, antibodies, receptors, nucleic acid). Recognition between the analyte and the bioelement generates, either directly or via a reaction product, a signal which is transmitted through a transducer (electrochemical, optical, piezoelectric, calorimetric). Finally, the signal is detected, measured, and processed to provide both qualitative and quantitative information

temperature gradient, which allows the melting curves of the two different amplicon sequences to be determined. Since these are sequence-specific, the two amplicons can be distinguished by their melting temperature. A SYBR Green-based triplex assay, allowing for the simultaneous detection of Maximizer 176, Bt11, and MON810 in GM maize, has recently been demonstrated to be effective in both seed and meal samples [43] with a limit of detection of 1%.

One of the major challenges for multiplex qRT-PCR is sensitivity and reproducibility, particularly minimizing the risk of false negatives. The complexity of the multiplex format means that optimization is more demanding than for uniplex platforms. An intra- and inter-laboratory validation of a duplex qRT-PCR screening method for GM detection based on the target sequences P-35S and T-Nos has been documented [63]. The primers and TaqMan probe sequences used were the same as had been validated in uniplex assays. The sensitivity of the duplex assay was comparable to that of the uniplex, in both cases 10 copies of P35S or of T-Nos were detectable. With a reference gene and relevant certified reference materials (CRMs) for quantification, the duplex method can be adapted to provide a semiquantitative estimate of the GM content of a sample. Both event-specific and species-specific qRT-PCR assays based on TaqMan® or similar chemistries have been duplexed by labelling each probe with a different fluorochrome [64]. The fluorophore double-stranded probes multiplex quantitative PCR (FDSP-MQPCR) method has been recently described [55] for the simultaneous detection of P-35S and T-Nos. In this application, the probes consist of two complementary oligonucleotides of different lengths. The longer strand is 5′ labelled with a fluorophore, and the shorter one 3′ labelled with a quencher, allowing close contact between the fluorophore and the quencher in the duplex state. In the absence of target, the probe remains double-stranded and does not fluoresce; in contrast, in the presence of a target, the short strand is displaced, allowing the fluorophore to be detected. In a test of ten soybean flour samples, FDSP-MQPCR gave quantitative results within 5 h, with accuracy [65] estimated to be at least 97.0% or higher over a range including from 0.5 to 5.0% GMO in standard materials.

The choice of reference materials for construction of calibration curves is an important issue in GM quantification. These can be prepared from known pure GM and GM-free material blended in known proportions. Such certified reference material (CRM) is restricted to only a few crop species, specifically maize, soybean, rapeseed, cotton, potato, and sugar beet. Reference materials are available covering GM concentrations of 0%, 0.1%, 0.5%, 1%, 2%, 5% for maize Bt-176, Bt11, MON 810, NK603, and for soybean RR; 0%, 0.1%, 0.5%, 1%, 1.7%, 4.3% for maize GA21; 0%, 0.1%, 1%, 10% for maize MIR604; DAS-59122; TC1507. A 1% reference material is available for cotton 281–24–236×3006–210–23, rapeseed GT73, GS40/90, MS8xRf3, and Oxy235, and potato EH92–527–1, as is a 0% and 100% reference for sugar beet H7–1.

Following the EU Commission recommendation 2004/787/EC in which the measurement unit is defined as the percentage of the number of GM target DNA sequences per target taxon-specific sequences calculated in terms of haploid genomes, plasmids certified on the basis of copy number could be used as alternative calibrants. Plasmids engineered to contain both the diagnostic marker and the endogenous gene have been developed. The analytical equivalence, commutability, of plant genomic DNA (gDNA) and plasmid DNA (pDNA) have been established in different laboratories [63, 66–68]. Commutability is defined as the similarity between the analytical response of a certain material and the response of routine samples [69]. The pDNA developed by Burns and colleagues [67] resulted to be useful as calibrants and allowed one to obtain a closer estimate of the GM percentage content of the samples analyzed, and also exhibited less variation with respect to the use of gDNA. Engel et al. [70] have developed synthetic DNA standards which contain targets specific for a GMO event and for the taxon-specific reference gene in a 1:1 ratio. These have been obtained through an amplification procedure which generates hybrid molecules.

Another important issue related to the preparation of reference material for GM quantification based on the haploid genome percentage, relates to the genetic structure of the analyte. Interesting research has been carried out by Zhang and collaborators [71] using MON810 maize kernels as an example to illustrate the impact of biological factors (zigosity, ploidy of tissue, and parental origin of the transgenic allele) on real-time PCR-based GM quantifica-

tion. Maize kernel is composed of endosperm (80–90% of the total weight), a small embryo, and a tegument of maternal origin. The endosperm is triploid, resulting by the fusion of two maternal nuclei and one sperm nucleus, and contributes from 36 to 59% of the total DNA; the embryo is diploid, resulting from the fusion of one haploid maternal nucleus and one haploid male nucleus, and contributes from 38 to 62% of total DNA; the tegument is diploid of maternal origin and contributes no more than 4% total DNA. Considering the complexity of maize kernel genetic structure, equations have been developed in order to calculate the correct number of GM haploid genome during calibration curve preparation. The correction factors proposed are particularly necessary when hemizygous GM material is used for calibration curves as in the case of the MON810 CRM IRMM-413 serials (ERM-BF413 [72]).

Analytical methods according to legislation

Under EC regulation 882/2004, all analytical methods used within the EU to control food and feed must be validated by the Community Reference Laboratory (CRL). Regulations 1829/2003 and 641/2004 stipulate that validated methods must be available for GMO detection, before authorization can be given to market food or feed containing GM material in the EU. Thus any request for authorization must also be accompanied by the proposed validation of the method, unless this method has already been validated. Where possible, the analytical method should be event-specific, and must include a description of the means for the isolation and purification of the analytes from the raw material. Since GMO detection methods involve multiple steps, from sampling to DNA extraction to PCR analysis, a modular approach is usually followed, in which the entire procedure is validated module-by-module, so that, once validated, all the modules can be combined in a flexible manner [73]. Modularity also allows separate improvement of the different modules based on direct experience and targeted validation tests. Currently, the EC project Co-Extra is pursuing the application of the modular approach and its critical evaluation in real-life situations.

Reference laboratory and method validation

The International Organization for Standardization (ISO) document 24276:2006 specifies the general requirements and definitions for methods for GMO detection. It focuses mainly on PCR-based methods, and details the guidelines for achieving validation. In the EU, validation is overseen by the Community Reference Laboratory for GM Food and Feed (CRL-GMFF), at the Joint Research Centre of the European Commission, Biotechnology & GMO Unit, established by regulation 1829/2003. The internet site (http://gmo-crl.jrc.it/statusofdoss.htm) lists the validation status for submitted protocols, and currently lists about 25 validated methods targeted to specific events in maize, rice, sugar beet, and cotton. First, the documentation provided by the applicant is assessed, then the detection method is validated for samples and controls provided by the applicant. The process is completed within 6 months, in cooperation with the partner laboratories of the European Network of GMO laboratories (ENGL), and outcomes are presented to the European Food Safety Authority (EFSA) and published on the EFSA web site (http://www.efsa.europa.eu). JRC and EFSA are cooperating in the definition of rigorous procedures for interpretation of the results in validation tests. Their approach based on fuzzy logic aims to summarize in a comprehensive indicator the performance of a method to facilitate comparisons and rankings [74].

Recently, Dong et al. [75] have established and published a web-based database containing details on all the published detection methods based on DNA and protein analysis, irrespective of their validation status. The database provides experimental details and primer/probe sequences.

Biosensors

The demand for DNA-based methods for GM detection has encouraged the development of sensor technology, which promises to generate results more quickly and is less technically demanding than PCR. Sensors are already employed to monitor various processes in food production, and their deployment has succeeded in improving quality controls throughout the food supply chain. Control of processing, quality assurance, and detection of problems are performed both with off-line and with in-line sensors ([76] and references therein). Simple sensors can be used to monitor readily measurable variables such as pH, temperature, viscosity, and sugar content. The principle of a sensor is that a specific change in the probe environment generates a change in a physical property of the probe (e.g., its electrical conductivity or its refractive index) and this change can be converted into an electrical signal in real-time [77, 78]. The European Commission has already financed over 20 research projects aiming to develop sensors for food analysis within the 4th and 5th framework programmes [79]. Biosensors are specifically sensors which carry an element(s) derived from a biological recognition system for the analyte, coupled to a means of converting and transmitting the signal (Fig. 3) [77, 80]. The bioelement can be a complex structure, such as a particular tissue or organelle, or can be composed of isolated molecules such as antibodies, enzymes, or nucleic acid [81]. Biosensors can

be highly specific to a particular analyte, but need also to respond in a way such that the intensity of the signal produced is proportional to the amount of analyte present [82]. Biosensor assays in which the analyte binds directly to the target are said to be "direct"; for these, the specificity of the interaction between sensor and analyte guarantees that any signal is specific. "Indirect" assays, in contrast, are those where the binding between analyte and bioelement is recognized via a coupled reaction, which can be either competitive or noncompetitive. The signals generated by sensors, and biosensors can be electrochemical, optical, piezoelectric, and calorimetric in nature [82]. The specificity of DNA-based sensors relies on sequence complementarity, as recognized by a hybridization assay, and requires that the sensor DNA be immobilized on the surface of the transducer element [83]. Recent reviews have addressed the employment of sensors and microarrays for GMO detection, in comparison with PCR-based methods [84, 85], stressing their high potential for improving analyses in particular conditions. Even though biosensors will not be able to replace the use of quantitative PCR for accurate determination of GMO amounts in products, they can nonetheless be useful in preliminary stages of control to identify samples to be subjected to successive analyses.

Electrochemical sensors

One application of electrochemical sensors for GMO analysis targeted the CryIA(b) gene [86]. The target was first PCR amplified using one thiolated and one biotinylated primer, unincorporated primers were removed by S1 nuclease treatment, the amplicons were purified by exposure to magnetic ferrocene–streptavidin, and were then bound to a gold electrode surface via their thiol moiety. In the presence of Hoechst 33258 (bisbenzimide) faclitating aggregation of DNA on the electrode surface, the double-stranded DNA produced an electrochemical signal which was detected by linear sweep voltammetry. Estimation of target copy number in the sample was based on signal size, in conjunction with a pre-measured reference curve. The system was able to detect standard materials at 0.9% threshold GMO content with an accuracy of 96%, by measuring the anodic current peaks generated by amplified CryIA(b) and SSIIb gene as reference. In a similar approach [87], a 25mer DNA sequence (part of T-Nos) was immobilized on the surface of a screen-printed carbon electrode. The electrode was then dipped into a solution containing either oligonucleotides or denatured target amplicons to allow hybridization to occur; this was then immersed in methylene blue, a dye which interacts with guanine accumulating on the electrode. An electrical signal was measured using square wave voltammetry, and the difference in signal strength between the hybridized targets (low) and the probe covered electrode (high) was found to be proportional to the quantity of target up to a target concentration of 10 mg/l. In a more recent approach [88] single-stranded DNA probes complementary to the 35S promoter or to the NOS terminator were immobilised on a gold electrode and challenged with DNA from Roundup Ready soybean, without prior PCR amplification of targets. The signal was detected as a reduction of current when methylene blue reacted with guanines. In a similar approach, detection was achieved using oligonucleotide probes bound with thiol to screen-printed gold electrodes, in conjunction with biotinylated target sequences and streptavidin–alkaline phosphatase [89]. The latter converts α-naphthyl phosphate to α-naphthol, which is electroactive and so can be detected via pulse voltammetry. In this case the signal strength increased in the hybridized state. The same authors have improved their "genosensor" by obtaining as product of the alkaline phosphatase reaction an insulating substance which could be assessed by faradic impedance spectroscopy [90]. The response of the sensor was reported to be linear over three orders of magnitude (12 pmol/l to 12 nmol/l). It was challenged with a 195-bp amplified target from the 35S promoter leading to a detection limit of 86 pmol/l, corresponding to 5.2×10^{8} copies of target in 10 μl.

Surface plasmon resonance (SPR) sensors

SPR is an optical-type sensor in which target recognition induces a modification in the refractive index at the metal/liquid interface, a change which is recognized as a shift in the resonance, as measured optically [91]. This therefore represents a system which avoids the need to label probes or analytes. It has been associated with a high level of sensitivity, accuracy, and precision, and has good reproducibility, and low limits of detection. In conjunction with DNA probes, SPR-based sensors can be used for direct detection, in sandwich assays, and for inhibition assays. In the direct mode, the analyte reacts with the recognition element (an antibody or a DNA probe) fixed to the sensor surface. SPR has been used for GMO identification [92], and have also found applications in the detection of pesticides, aflatoxins, bacterial toxins, and pathogens in food [91]. BIA core is a commercial SPR-based biosensor. In this system, plane polarized light is reflected from a gold-coated sensor chip when the target molecular interaction occurs. SPR results from the extinction of reflected light at a known angle (the "SPR angle") which is determined by the refractive index of the solution. When the ligand binds to the chip, the refraction index changes the SPR angle, and this change can be sensed. The introduction of the analyte generates a further change in the resonance, provided that the ligand interacts with the

analyte. The sensor chip can be regenerated by chemical removal of the bound analyte. Since the change in refractive index is proportional to the adsorbed mass, there is potential for the real-time quantification of the ligand–analyte interaction, in the same way as for nucleic acid hybridization. The BIA core system has enjoyed a wide spectrum of detection uses, including GMO analysis [93–95].

Quartz crystal microbalance (QCM) sensors

QCM sensors consist of a quartz layer surface coated with the biorecognition element; when molecules are absorbed to this surface, the mass of the unit is increased and this induces a change in the oscillation frequency of the crystal when stimulated by an electric field. This change can be converted into a conventional electrical signal. QCM sensors have been used to detect the presence of specific pathogens in both water and food [80]. For GMO detection, the recognition element would consist of a DNA probe(s), able to hybridize with complementary target present in the analyte. Probes complementary to P-35S and T-Nos have been immobilized to the quartz using either thiol groups [96], or via the avidin–biotin interaction [97]. A QCM sensor carrying a probe for the CryIA(b) gene has been shown to be an effective detector of the Bt transgene in biscuits and reference materials, and the linear dependence between the resonance frequency and GM content implies that this platform could be developed for quantitative assays [97]. A 21mer probe complementary to the EPSPS gene conferring resistance to the herbicide glyphosate was biotinylated and immobilized via avidin binding to the gold surface of a QCM sensor [98]. The binding of an amplified fragment of EPSPS decreased the frequency of oscillation, whereas noncomplementary molecules did not generate any change. The sensor responded to the presence of EPSPS without the need for a prior PCR amplification, so that feed samples containing 30% RR soybean (equivalent to about 5×10^5 gene copies present in the sensor cell) could be detected. Amplification of the target would decrease this LOD. This QCM sensor can be re-utilized many times, up to 20, with an analysis time of 15 min [99].

Lab-on-a-chip

Nanotechnology and microfluidic principles are being applied to the design of microdevices and microsystems for analytical purposes [80]. Both food safety and quality can be addressed with these technologies, especially in the context of arrays of sensors aimed at the simultaneous detection of multiple targets. In channels with a diameter of a few micrometres, fluid flow is governed by properties which can be exploited for analytical purposes (separations coupled to mass spectroscopy, high-throughput screening in drug development, bioanalyses, examination and manipulation of samples consisting of a single cell or a single molecule) [100].

The generic requirements for these devices are a system to inject reagents and samples, a system to move and mix these, and a set of detectors (and/or components for the purification of particular products). Proper mixing is particularly important, because the laminar flow of fluids in a microchannel is turbulence-free. The effectiveness of LabChip™ technology to detect GM content in soybean has been compared with conventional electrophoresis and with capillary electrophoresis using the Bioanalyser 2100 (Agilent Technologies). While both the latter techniques were reliable, the LabChip™ approach gave more reproducible results [101]. The advantages of biosensors in food analyses are clear. They can measure different analytes in parallel; they are versatile, highly sensitive and specific; they can operate in real-time and in situ; they can be miniaturized, and assembled into arrays to make portable devices; and they do not require highly trained personnel for their operation.

Several innovative approaches are currently under development for rapid and automatic detection of biological warfare agents, such as disease organisms. The development of portable instruments for real-time PCR and for microarrays based on microfluidics will likely benefit the food industry, since all these systems can be adapted to detection of food ingredients and contaminants, including GMOs, simply by changing the probes employed [102].

Array-based methods

An increasing number of GM crops are being developed, and are receiving market approval, so it is of some importance to elaborate methods which can identify several transgenes in a single reaction. As a result, multiplex PCR has become the prime tool for GM detection [103]. As the level of multiplexing necessary rises, it will become ever more difficult to distinguish between the various PCR products on the basis of their amplicon length. The development of a more flexible discriminating tool than conventional electrophoresis is therefore of some priority if an ever broader range of GM crop varieties are to be efficiently genotyped [104]. The most promising alternative discrimination platform at present is the microarray, since this is designed a priori as a means to screen many targets in parallel within a single sample. Thus, the microarray has the potential to combine detection, identification, and quantification of effectively an unlimited number of GM events in a single experiment [105]. Since the microarray provides a systematic means to survey DNA and RNA variation it has become a ubiquitous tool in both molecular biology research and clinical diagnostics. As a result,

substantial investments has been made in this technology in both the public and private sectors [106].

The principle of the microarray relies on the key insight, made already over a quarter of a century ago, that labelled nucleic acid molecules can be exploited to probe other nucleic acid molecules attached to a solid support. Each microarray consists of a solid support (often glass) upon which has been deposited ("printed") a large number of discrete aliquots of known nucleotide sequences ("features"), which recognize their target via base complementarity [107]. In the context of GM detection, a number of the features will have been derived from transgene sequences, so the pattern of hybridization (both qualitative and quantitative) will reveal both whether the analyte represents a GM variety, and which GM events are present. The microarray format may possibly be adaptable for protein-based detection, with the features represented by antibodies or other selective proteins [108] though in a short period there will never be large numbers of antibodies or selective proteins for such an application.

The conventional solid-phase array has some important disadvantages. One is that the analyte solution is static on the array during hybridization and each arrayed spot is in effect sampling from its immediate or near immediate environment. A second is that the high concentration of DNA present at each feature can generate steric hindrance during the hybridization reaction. Several alternative array systems are currently under development to overcome these shortcomings. An example is the Nanogen system, in which the probability of successful hybridization events is enhanced by attaching a negative electric charge to the analyte, and providing the features with a positive charge [http://www.nanogen.com/technologies/microarray/]. In gel-based DNA chips, steric hindrance is reduced because the surface over which hybridization occurs is three-dimensional. A rather different approach dispenses altogether with the concept of a solid support, and instead attaches the probes to microspheres, which are held in a liquid suspension (e.g., suspension array technology [109] and bead array counter [110]). The large numbers of features presented on the microarray imply a large data output, so the design and provision of adequate data analysis instrumentation are becoming increasingly important. This is an active area of product development, driven by the interest in gene expression profiling, where analysis of large data sets is already routine [108].

A new microarray tool was developed by Tengs et al. [34]. The method is PCR-independent and applies direct hybridization of total genomic DNA. Using custom-designed microarrays (NimbleExpress arrays, Affymetrix, Santa Clara, CA, USA), they analyzed genetically modified lines of *Arabidopsis thaliana* and *Oryza sativa* showing that without prior knowledge about the transgene sequence, fragment(s) (≥140 bp) of the element(s) used in the genetic transformation can be identified. These arrays were designed to have 25 basepair probes tiled throughout 235 vector sequences downloaded from GenBank. This approach gave good results in detecting specifically and in a very sensitive way the presence of transgene sequences and gave sufficient information for further characterization of unknown genetic constructs in plants. The only requirements were access to a small amount of pure transgene plant material; genetic construct above a certain size (here ≥ 140 bp); and construct showing some degree of sequence similarity with published genetic elements.

Microarray technology can be combined with multiplex PCR, for instance, to assess the content of various transgenic maize events in samples of food and feed by using the multiplex PCR amplicon as the analyte to hybridize to a DNA array carrying transgenic features [111]. A low density array allowing the parallel detection of nine GM events, including P-35S, T-Nos, and nptII, used biotin labelled amplicons, which were detected colorimetrically [112]. A further low density array has been described [113] which detects P-35S and T-Nos, as well as corn invertase and soy lectin genes. This array employed a microporous, hydrophobic polyester cloth as a solid support. Similarly, an array containing features based on eight structural genes has been demonstrated to be informative for the identification of RR soybean [114], and this has recently been extended to include 20 genetic elements [104]. This system cannot only tell whether the sample is made of GMOs, but it can also distinguish which kind of plant it belongs to and which characteristics it has like insect- and herbicide-resistance [104]. Specific transgene integration junction sequences were exploited as features to identify one commercial GM-soybean and six GM-maize events [103].

A commercial kit, DualChip®GMO, has been developed by Eppendorf Array Technologies (EAT, Namur, Belgium) by coupling multiplex PCR assays to microarray hybridization. The system detects and identifies genetically modified (GM) events by screening simultaneously multiple genetic elements. The experimental design consists of four sets of multiplex polymerase chain reaction (PCR) using biotinylated primers, specific for the amplification of screening elements, species reference elements, and control elements. After multiplex amplification the PCR products are hybridized on one single microarray containing capture probe-sets which are specific for the sequences present in Bt-176 Maize, Mon 810 Maize, Bt-11 Sweet Maize, Mon 531 cotton, GA21 Maize, and Roundup Ready TM Soya GMO events. The detection of the biotinylated sequences hybridized is done using the Silverquant colorimetric detection.

Using a rather different approach, the ligation detection reaction (LDR) has been coupled with an universal array technology to identify and quantify the cryIA(b) gene from

Bt176 maize [115]. The same strategy was also able to simultaneously detect five transgenic events and two endogenous controls (soy lectin and maize zein) in food samples, following two multiplex amplification reactions [38, 116].

PNAs have been proposed to be superior to oligonucleotides as the basis for microarray features, because their hybridization characteristics are more robust [117]. Several PNA-based arrays have been used to identify DNA mutations [118], and recently a PNA chip has been developed for the parallel detection of five transgenes and two plant species in both raw material and processed food [36]. The protocol for attaching the PNA probes to the slide was modified from that used for oligonucleotides, with spacers added to distance the features from the slide surface. The combination of this array platform with multiplex PCR appears to represent a reliable analytical means for GMO detection in the food chain [36].

Concluding remarks

A diversity of methods and strategies has been brought to bear on the issue of GMO detection. Most of these are based on PCR, either simplex or multiplex, with some using a real-time format. The advantages of analysis at the DNA level, rather than at the gene product level, have been demonstrated by the outputs of various research projects financed by the EC, starting from FP5 (http://cordis.europa.eu/fp5/projects.htm): GMOCHIPS, QPCRGMOFOOD, DNA-TRACK, GMOBILITY, ENTRANSFOOD. A current EC project, Co-Extra (www.coextra.eu), is studying the coexistence and traceability of GMOs, specifically targeting sampling, statistics, method development, and the development of a modular approach to validation. Much of the debate over the labelling of GMO-derived or GMO-containing products, in both the legislative and the scientific arenas, concerns the accuracy of analytical methods. In order to be specific, a method must target a unique feature of the GM event, and must be able to detect all known authorized and unauthorized events.

The CRL-GMFF publishes validated protocols for the analysis of authorized GM crops. Several issues still remain unresolved, specifically:

- The definition and identification of endogenous genes for quantification
- Assays for non-authorized GM events, usually because relevant DNA sequence information is lacking
- Assays for GM crops carrying stacked transgenes, specifically how to differentiate these from mixtures of single events
- The quantification of GM material, considering variation in genome size and tissue ploidy level
- Optimizing methods for DNA extraction from different food matrices
- Sampling and statistical analysis
- The development of field-based analyses, using portable instrumentation

Some of the cutting edge methods described in this paper represent steps towards the solution of a number of these problems.

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