From Sampling to Quantification: Developments and Harmonisation of Procedures for GMO Testing in the European Union

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Abstract

The objective of this review is to provide an overview of the scientific background and of the legal context defining the framework of genetically modified organism (GMO) testing across Europe. Specifically, we provide a comprehensive summary of the procedures for GMO testing, starting from sampling to final quantification of GMO content, including an overview of the legislative frame regulating such procedures, and a summary of the actions taken at the European level to ensure harmonisation of GMO testing among Member States. Some topics that have been the object of scientific debate over the past years will be carefully addressed and the results of the scientific experience gained reported. Not all of the issues raised have been fully resolved even though remarkable progress has been made with the reaching of commonly agreed definitions and applicative procedures. There are still some aspects under discussion, making the GMO issue an ongoing, interesting and continually evolving topic.

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Riassunto
Scopo di questa recensione è fornire il quadro della situazione scientifica e del contesto legale entro il quale vengono definite le procedure per l'analisi degli organismi geneticamente modificati (OGM) in Europa. In particolare, viene fornito un ampio sommario delle metodiche utilizzate per analizzare gli OGM partendo dal campionamento fino alla quantificazione finale del contenuto di OGM nel campione, includendo una panoramica del quadro legislativo che regola tali procedure, ed un sommario delle azioni intrapprese a livello europeo per assicurare l'armonizzazione delle procedure per l'analisi degli OGM nei Paesi Membri. Alcuni argomenti che sono stati oggetto di dibattito scientifico negli anni passati verranno analizzati attentamente e verranno riportati i risultati dell'esperienza scientifica acquisita. Non tutti i problemi sollevati sono stati pienamente risolti, anche se sono stati fatti progressi notevoli con il raggiungimento di definizioni e procedure applicative comunemente accettate. Ci sono ancora alcuni aspetti in discussione, e questo fa degli OGM un tema interessante in continua evoluzione.

1. INTRODUCTION

Genetically modified organisms (GMOs) are defined in the EU legislation as '*organisms, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination*' (European Commission, 2001).

Since the first experiments on tobacco in the 1980's, several plants species, including both monocots and dicots (Birch, 1997), have been genetically modified to improve specific characteristics (e.g. yield, quality, pest resistance), by adding one or more useful character previously not present in the non-modified counterparts. Such changes are generally obtained by the insertion of one or more genes using a range of available techniques defined as "gene transfer technologies", reviewed in Hansen and Wright (1999) and discussed in detail in a number of papers (Gelvin, 2000; Tzfira and Citovsky, 2002; Van den Eede *et al.*, 2004). In practice, plant transformation involves the insertion of a piece of DNA (the insert) into the genome of a target organism. The insert is a combination of several small pieces of DNA. In its easiest format the insert is composed of a promoter, a coding sequence, and a terminator. All together, these pieces form a 'gene cassette' or construct. In practice, constructs are complex structures, containing two or more gene cassettes. The successful result of plant transformation is the presence of a new fragment of DNA (the insert) into the receiving plant genome conferring one or more new trait/s to the host organism.

From a practical point of view, the possibility to discriminate if a plant, a seed or a derived product has been genetically modified requires analytical methods capable of recognising either the foreign DNA introduced, or the corresponding encoded protein/s. Analytical methods most widely used for this purpose fall into two categories: 'DNA-based methods', that target the DNA that has been physically inserted during transformation, and 'protein-based methods' that identify the presence of newly synthesised proteins by exploiting the specificity of binding between an expressed antigen and the associated antibody (Ahmed, 2002; Anklam *et al.*, 2002; Holst-Jensen and Berdal, 2003; Miraglia *et al.*, 2004).

From the legal viewpoint, the use of GMOs, their release into the environment, their cultivation, importation and, in particular, their utilisation as food, food ingredients and animal feed, is regulated in the European Union by a set of strict rules. These rules guarantee a high level of protection to human and animal health and to the environment, whilst ensuring the effective functioning of internal markets. A key technical element for the authorisation of GMOs within the EU is the provision of an

event-specific quantitative detection method to allow the control and monitoring of the GM event in the distribution chain, i.e. a method that can unambiguously distinguish and quantify the GMO of interest (i.e. the event) from all other possible GMOs and non-GMOs. Such a method should also be validated according to internationally accepted standards, in order to be proven as fit for regulatory purposes.

Several textbooks (Sambrook *et al.*, 1989; Innis *et al.*, 1990), reviews (Wolcott, 1992; Carrino and Lee, 1995; Miraglia *et al.*, 2004) and ad hoc training material (Querci *et al.*, 2004) provide detailed scientific and technical information on the methodologies currently available for the detection and quantification of biological targets such as GMOs. The objective of the present review is to provide a comprehensive summary of the procedures for GMO testing in the EU, from sampling to final quantification, and an overview of the legislative frame regulating such procedures. Particular attention will be given to those topics that have been the object of scientific debate over the past several years, and to the scientific experience gained as a result of these international discussions. As it will become clear upon reading, not all of the recently raised issues have been fully resolved (see Cellini *et al.*, 2004 and Miraglia *et al.*, 2004 for in depth overviews). Although tremendous improvements have allowed the reaching of commonly agreed definitions and applicative procedures, there still remain aspects under discussion and debate, making the GMO issue an ongoing, interesting and continually evolving topic.

2. EU REGULATORY FRAMEWORK

The legal frame regulating GMOs and GMO-derived products in the EU consists of horizontal (background legislative framework) and vertical (sector-specific) legislation, designed to provide a set of legislative tools according to the specific needs and requirements of each sector involved. The first community legal instruments were produced in the 1990's with the specific aim of creating a unified approach to protecting human and animal health, and the environment. In particular, two directives defined the horizontal legislative framework: Council Directive 90/219/EEC, concerning the contained use of GMOs (European Commission, 1990a), amended in 1998 by Council Directive 98/81/EC (European Commission, 1998a), and Council Directive 90/220/EEC on the deliberate release into the environment of genetically modified organisms (European Commission, 1990b), recently repealed by Directive 2001/18/EC (European Commission, 2001). Directive 2001/18/EC, which entered into force on 17 October 2002, puts in place a step-by-step process to assess

possible environmental and health risks on a case-by-case basis, before granting the authorisations for the experimental release (part B of the Directive) and marketing (part C of the Directive) of GMOs. Directive 2001/18/EC upgrades Directive 90/220/EEC mainly by expanding public information requirements, by introducing general rules for traceability and labelling, and by strengthening the decision-making process to assess possible environmental risks linked to the release of GMOs. Approval is given for a maximum period of ten years starting from the date when the consent is issued, and it can be renewed. Consents already granted under Directive 90/220/EEC had to be renewed in order to avoid disparities and to ensure compliance with Directive 2001/18/EC requirements. Following the placing on the market of a GMO, the notifier is the legal body responsible for ensuring that monitoring and reporting are carried out according to the conditions specified in the consent.

In addition to the horizontal Directives, a series of vertical legal instruments, dealing specifically with the approval and safe use of GMOs intended for human and animal consumption, have been developed over time (European Commission 1997, 1998b, 2000). Finally, in April 2004, two Regulations amending or repealing previous legal instruments entered into force: Regulation (EC) 1829/2003 on genetically modified 'food and feed', and Regulation (EC) 1830/2003 on 'traceability and labelling of GMOs in the EU. Regulation (EC) 1829/2003 (European Commission, 2003a) implements the "*one key-one door*" principle, meaning that a centralised procedure covers all applications for placing on the market, whether they concern the GMO itself or the food and feed products derived thereof. A single authorisation covers both food and feed use, therefore fulfilling the legal vacuum for the approval of feed products, which existed before the entrance into force of this Regulation. According to Regulation (EC) 1829/2003, the applicant must submit a full dossier, inclusive of a detection method for the GMO pending approval. The dossier is evaluated by the European Food Safety Authority (EFSA) and the proposed detection method is validated by the Community Reference Laboratory for GM Food and Feed (CRL-GMFF), established within the EC's Joint Research Centre[1]. As for Directive 2001/18/EC, authorisations are limited to ten years, with the possibility of renewal. The 'food and feed' Regulation reduces the *de minimis* thresholds for labelling from 1%, as previously defined in Regulation (EC) 49/2000 (European Commission, 2000), to 0.9%. This threshold is calculated on

[1] *The CRL-GMFF is assisted by a consortium of more than 100 national reference laboratories that form the European Network of GMO Laboratories (ENGL, http://biotech.jrc.it).*

individual ingredient level (Figure 1). An additional 0.5% tolerance level has been introduced as a transitional measure for non-approved GMOs, provided that their presence is adventitious and that the specific GMO under consideration has already a favourable opinion from the European Community Authority.

Since 1997, the labelling of products containing GMOs is mandatory under Regulation (EC) 258/97 (European Commission, 1997), and Regulation (EC) 1830/2003 (European Commission, 2003b) reinforces the already existing labelling rules by extending mandatory labelling to all food and feed products derived from GMOs, including those in which GMO-derived DNA or proteins are no longer detectable (e.g. highly refined oils). Regulation (EC) 1830/2003 requires traceability at all stages of the placing on the market, the assignment of a unique identifier or "code" for each GMO, further defined in Regulation (EC) 65/2004 (European Commission, 2004a), and the establishment of a central register. The central register was established as a tool to help Member States meet the requirements on inspection and control measures; it contains all available sequencing information and reference material for authorised GMOs and, where available, relevant information concerning GMOs not authorised in the European Union.

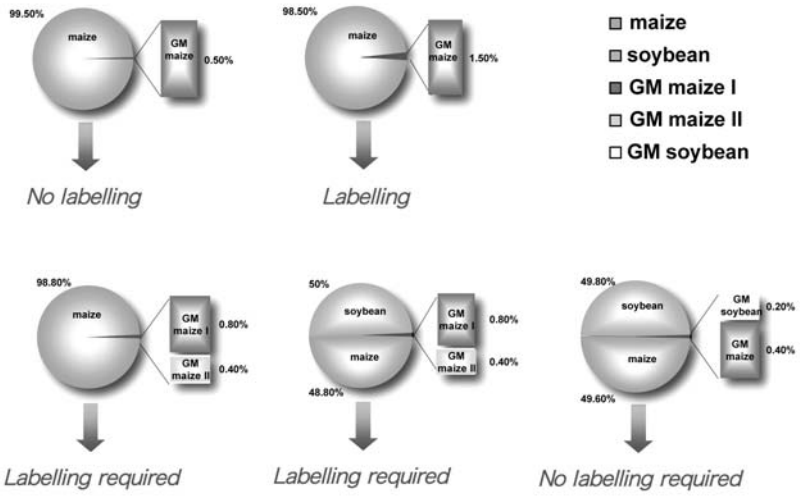

Figure 1. *Examples of labelling requirements in Europe according to Regulation (EC) No 1829/2003*

3. INTRODUCTION TO GMO ANALYSIS – FACTORS INVOLVED

3.1. Sampling

As described above, current EU legislation requires the labelling of food products, raw or processed, which contain GM material. This requirement makes the definition of appropriate sampling protocols for GMO detection and quantification one of the most critical aspects of GMO control in market products. Indeed, according to Lischer (2001): "*the effort of the analyst in the laboratory is futile if sampling has not been carried out correctly. Analytical reliability is today limited not by the analyst intrinsic qualities, but by the lack of reliability of the samples submitted to the analytical process. Quality estimation is a chain and sampling is by far, its weakest link*". Consequently, large-scale testing and monitoring programs are being conceived and executed in order to check for compliance with the regulations involved. This leads to a strong interest in the schemes adopted for the sampling of food products, to ensure accuracy and precision of GM testing surveys.

In general, the distribution of a contaminant in a bulk mass greatly affects the effectiveness of sampling procedures (Cochran, 1977; Binns *et al*., 2000). In particular, among all currently used sampling guidelines for the testing for GMOs only two, Commission Recommendation 2004/787/EC (European Commission 2004c) and prCEN/TS 21568 (CEN, 2005) were specifically developed for GMO surveys and are free from distributional assumptions. Others that were originally developed for quality control and merely adopted for GMOs (Kay, 2002), have stringent distribution requirements (Paoletti *et al*. 2003a) and are not applicable in cases of heterogeneity (Kay, 2002). Specifically, all of these protocols are based upon the assumption that GM material, if present, is randomly distributed. Under such an assumption, the mean, the standard deviation, and both the producer and consumer risks can be estimated using either Binomial or Poisson distribution (Remund *et al*., 2001 for GMO discussion; Binns *et al*., 2000 and Cochran, 1977 for specific statistical discussions). Attempts to adapt the mathematical properties of Poisson distributions to non-random situations have been made (Hübner *et al*., 2001). However, the statistical appropriateness of such approaches is dubious because of the violation of inherent assumptions (e.g. normal variance characteristics) required for the use of such tools.

A population (lot) of particulate material is always affected by a certain amount of heterogeneity. According to Lischer (2001), as homogeneity is the zero of heterogeneity, a random population is the zero of a chronological series. Under this scenario, perfect randomness is a limiting case, which is unlikely to be realistic, especially once it is considered that opportunities for segregation

generally occur consistently in time and space, and that gravity causes vertical segregation during transportation and/or handling of particulate material. In addition, it has been shown that even modest deviations from randomness have a strong effect on the accuracy (contamination %) and precision (contamination variance %) of the contamination estimates (Paoletti *et al.*, 2003a). As a consequence, in the case of heterogeneous distributions, samples produced according to currently adopted sampling protocols have a high probability of incorrectly representing the lot.

From a practical point of view, the scenario becomes even more complex. Studies reporting estimates of GM contamination rates seldom provide details regarding the applied sampling procedure/s, and international standard sampling protocols adopted for GMO testing are often modified depending upon contingent needs and available facilities. In light of these theoretical and practical problems associated with sampling for GMOs, it is evident that research projects delineating the real-world distribution of GM materials in bulk commodities must be undertaken to ensure correct action with respect to the required distribution assumptions (e.g. randomness), and to develop more appropriate sampling plans based on the actual heterogeneities met.

Recently, we conducted a research project entitled "Kernel Lot Distribution Assessment" (KeLDA) in collaboration with eight laboratories from the European Network of GMO Laboratories (ENGL) from six different Member States. The KeLDA project (Paoletti *et al.*, 2006) was the first study assessing the distribution of GM material in soybean lots imported into the EU. Through the investigation of GM material distribution patterns in fifteen independent large soybean lots imported within the EU, KeLDA fulfilled two main tasks: a) verifying if the assumption of random distribution, implicit in the vast majority of the sampling protocols currently adopted for GMO analysis, is indeed violated in practice; b) estimating the amount of heterogeneity and spatial distribution patterns among lots that can be expected when sampling for GMO testing. This was particularly important with respect to the harmonisation of sampling procedures because it indicated the upper limit of inherent distribution assumption requirements that sampling protocols can have when applied to GMO analysis.

The KeLDA results (Paoletti *et al.*, 2006) are a milestone in the understanding of sampling for GMO testing because they provide the first documented evidences that GM material distribution within and among lots of bulk commodities show highly significant deviations from randomness. The unequivocal evidence that GM material distribution is heterogeneous highlighted the need to develop sampling protocols based on statistical models free of distribution assumption requirements, and capable of taking into account the specific

variability of spatial patterns that KeLDA has demonstrated.

To date there is only one distribution-free statistical model (KeSTE) of known properties developed to estimate sampling errors for GMO testing (Paoletti *et al*., 2003b). Although still under refinement, the model, upon which the sampling protocol suggested in the Commission Recommendation 2004/787/EC (European Commission, 2004c) is based, allows estimating the sampling error associated with different sampling protocols not only for GMOs, but also for any consignment of particulate material with respect to any kind of contamination. In particular, the model presents two novelties: first, freedom from any distribution assumption; second, the possibility of estimating the magnitude of the sampling error associated with different sampling protocols as a function of specific distributional properties. A specific description of the mathematical properties of the model can be found in Paoletti *et al*. (2003b), whilst a description of its application in practice, as presented in Commission Recommendation 2004/787/EC, is provided below.

The sampling protocol suggested in Commission Recommendation 2004/787/EC is recommended for lots of bulk agricultural commodities (e.g. grains) and for lots of non-packed food products (e.g. flour), whereas the International Seed Testing Association (ISTA) rules are recommended for seed lots, and sampling procedures described in ISO standard 2859 (ISO, 1999a) are recommended for lots of packed food products (e.g. cookies).

The sampling protocol for grains and non-packed food commodities is articulated into three steps:

Step 1: a bulk sample is created by mixing all of the increments collected from a given lot. The increments should be systematically sampled according to the principles described in ISO standards 6644 and 13690 (ISO, 2002; 1999b). In the case of flowing commodities, the sampling period should be defined as: total off-loading time/total number of increments. In the case of static sampling, increments should be collected at specific sampling points. Such sampling points should be uniformly distributed throughout the lot volume, according to the principles described in ISO 13690. The number of increments or sampling points is defined according to the lot size, as indicated in Table 1. At each sampling interval (systematic sampling of flowing material) or sampling point (static sampling), an increment of 1 Kg should be collected and split into two portions of 0.5 Kg: one to be used as an increment for the production of bulk sample, the other to be stored as a file increment sample for further analysis, if necessary (see below). The increments collected are then thoroughly mixed (according to the procedures described in ISO 13690 and 6644) to form the bulk sample.

Step 2: the bulk sample is used to produce an analytical sample according to the procedure described in ISO 13690 and ISO 6644, and its GMO content

Table 1. Number of increments or sampling points defined according to lot size as indicated in Commission Recommendation 2004/787/EC

Lot size in tonnes	Size of the bulk sample in kg	Number of increments
≤ *50*	5	10
100	10	20
250	25	50
≥ *500*	50	100

is evaluated with respect to legal thresholds. If the result of the analysis is close to the established threshold (threshold ± 50 % of its value), an estimation of the associated uncertainty is necessary (see step three of the sampling protocol below). If the result is low (< threshold – 50 % of its value) or high (> threshold + 50 % of its value), no further analysis is required and the lot can be considered as negative or positive, depending upon whether the analytical result is below or above the relevant threshold.

Step 3: an analysis of individual increments (file increments stored at the beginning of the procedure) to estimate the confidence interval around the estimate is carried out for those analytical samples derived from the bulk sample if the GMO content is close to the legal threshold (threshold ± 50 % of its value). Specifically, if there are 20 or fewer file increments, as in the case of small lots (see Table 1), all file increments should be analysed individually to allow an estimate of the uncertainty associated to the estimate. If instead there are more than 20 file increments, 20 samples should be randomly selected and individually analysed for the presence of GMOs. Analytical results of these 20 samples are used to estimate the uncertainty of the GMO content expressed as the standard deviation. If this uncertainty is acceptable, no additional analysis of the remaining file increments is necessary. If instead the level of uncertainty is too high, additional analysis of the remaining file increments is necessary. The number of additional file increments to be analysed is established on a case-by-case basis, depending upon the level of uncertainty estimated from the initial 20 samples. The process stops when either or both of the following is true: the estimated lot GMO content (mean GMO content of the analysed file increments) is above or below the established threshold ± 50 % of its value; the uncertainty (standard deviation) of the measured lot GMO content reaches an acceptable level (± 50 % of the mean analytical result).

3.2. Analytical approach

Plant material that has been genetically modified can be distinguished from

non-modified material because it contains either unique novel DNA sequences and/or unique novel proteins. Accordingly, analytical methods for the analysis of genetically modified plants and derived products can be divided into two categories relying either on the selective identification of specific nucleic acid sequences integrated into the genome through the transformation process, or on the specific immunological detection of the novel protein(s) synthesised by the newly introduced gene(s), respectively (Querci *et al.*, 2004). Both approaches have been and are widely used in GMO analysis, but the selection must be carefully evaluated since their application might be occasionally limited or hampered by the nature and characteristics of the target. It should be noted, for example, that while foreign DNA sequences, once integrated into the host plant genome, are stably maintained over generations following Mendelian rules, their expression level is not always sufficient for detection purposes (Longstaff *et al.*, 1995). Protein-based methods can therefore be used only when the insertion of foreign DNA leads to a sufficient quantity of specific new protein/s to ensure reliable detection. A description of both protein- and DNA-based methods, with a summary of their respective advantages and disadvantages, is provided below.

3.2.1. DNA–based analytical methods

Almost all currently available detection methods relying on the selective identification of specific nucleic acid sequences are based on techniques applying the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis and Faloona, 1987). PCR is an *in vitro* method for the enzymatic amplification of specific nucleic acid sequences. In brief, two primers are synthesised to be complementary to known sequences flanking the target sequence to be amplified on opposite strands, and oriented so that DNA synthesis proceeds across the region between the primers (Figure 2).

In theory, provided that the accepted rules for primer design are followed, any DNA sequence can be amplified by PCR. The principal and most important elements determining the level of specificity of a PCR assay are the primers, and more specifically the selection of elements to which the primers

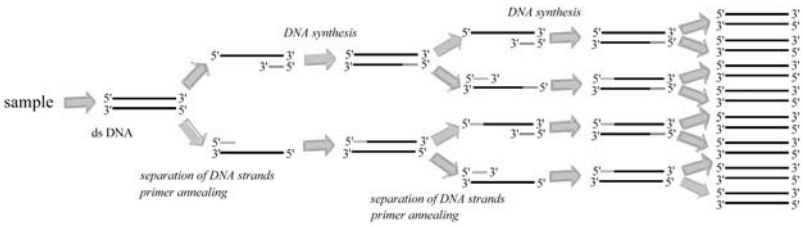

Figure 2. *Scheme of DNA amplification by the PCR process*

are directed (Figure 3). As a consequence, the availability of precise and comprehensive information is a prerequisite for the development of specific DNA-based detection methods. Data should include detailed information regarding the foreign DNA, the structural gene and regulatory elements integrated into the recipient genome and their nucleotide sequence.
"Broad range" PCR detection systems - generally called 'screening methods' - are obtained by designing primers specific to sequences that are commonly used in a variety of GMOs, e.g. the regulatory sequences (promoters and terminators). More "specific" PCR systems - gene-specific and construct-specific, respectively - can be designed by either targeting the structural genes introduced, or by choosing primers specific to DNA sequences located in different genetic elements (e.g. promoter-structural gene, structural gene-terminator). Finally, provided that the specific and complete sequence information is available, "line-specific" or "event-specific" systems can be developed by selecting a "unique" sequence combination, present only in that transformed line. This is generally obtained by designing primers hybridising in the DNA region spanning across the interface of the integration site. The junction between the inserted DNA and the host DNA offers a unique nucleotide sequence providing an ideal target for a highly

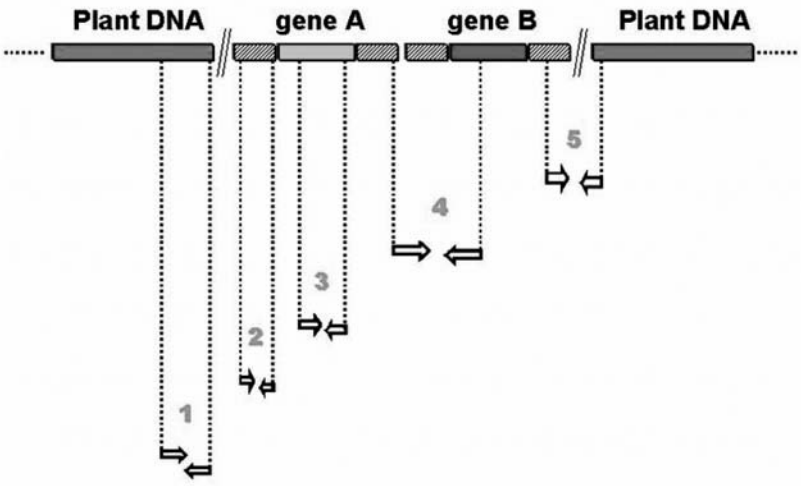

Figure 3. *Different target categories of PCR-based tests in GMO analysis. Increasing levels of specificity (from 1 to 5) are determined by the DNA selected as PCR target. 1. species-specific PCR; 2. screening PCR; 3. gene-specific PCR; 4. construct-specific PCR; and 5. event-specific PCR*

specific PCR test. Indeed, the insertion region is a unique identifier in each and every GM event since the integration of the construct in the plant genome occurs randomly (Thomas *et al.*, 1994; Jacobs *et al.*, 1995).
PCR methods with the different levels of specificity described above are employed regularly to answer to the sequential questions linked to the different steps of the procedure in place within the EU for GMO testing (Figure 4): in the first instance it is necessary to assess if the product contains any GMO, and a first "screening test" providing a general yes/no answer must be carried out. If the product is positive, then it is necessary to identify the specific GM line (or lines) to assure that it is (are) authorised in the European market. In order to answer this second question it is necessary to apply one or more specific methods allowing discrimination and identification. The third and final step is the quantification of the GM event(s), which can be obtained by applying a quantitative method. A detailed description for each of these three steps, screening, identification and quantification, is provided below.

3.2.1.1. Screening and Identification

The screening and identification steps are commonly performed using conventional or so-called "end point" PCR protocols. As already indicated, PCR is an *in vitro* method for the enzymatic amplification of specific nucleic acid sequences based on the mechanism of DNA replication, where DNA is unwound to single-stranded DNA, duplicated and rewound. The repetition of these basic

Figure 4. *Sequential questions and corresponding analytical steps for GMO testing according to EU legislation*

steps - composing a PCR cycle - allows the million-fold synthesis - or amplification - of the specific target DNA sequence. In conventional or "end-point" PCR protocols, the result is analysed at the end of the amplification process and the visualisation of the PCR product is carried out by agarose gel electrophoresis.
Even if, in theory, PCR allows the doubling of the amount of target DNA at each cycle, in real life PCR reactions never reach the theoretical 100% doubling efficiency. The reaction efficiency has indeed a significant impact on the final number of DNA molecules and it depends on the characteristics of the target DNA, on the level of optimisation of the reaction, and on other intrinsic kinetic factors. Three phases can be identified in a typical curve representing the progression of a PCR reaction over time: an initial lag phase, an exponential phase characterised by the exponential accumulation of DNA molecules, and a final plateau. PCR protocols typically consist of a 30 to 40-fold repetition of the 3-step PCR cycle and the amount of PCR product accumulated at the end of the amplification process – usually at the plateau phase - does not correlate with the initial amount of DNA template. The gathered information is therefore only qualitative (presence / absence). The positive result is therefore visualized on a gel as a single sharp band with a size determined by the distance of the two primers on the target DNA molecule.
Screening methods, developed by designing primers targeting the most common sequences used in transformation, are meant to detect the widest range of GM crops (lines) without requirements of precise identification. From a practical view point, screening methods are useful for the rapid and reliable reduction of test samples by direct identification of negative samples, which do not need to be further analysed.
To date, only a limited number of well-characterised regulatory elements (promoters and terminators) are employed for GMOs development, the most frequently of which originate from *Agrobacterium tumefaciens* and Cauliflower Mosaic Virus (CaMV) (Hemmer, 1997). It is reported that out of 64 analysed transgenic crops, 60 contained at least one genetic element derived from these two organisms (BATS, 2003). The first internationally validated screening methods, based on the detection of the CaMV 35S promoter and of the nos terminator from *A. tumefaciens*, were developed by Lipp *et al*. (1999, 2001) for the analysis of Roundup Ready® soybean and Maximizer maize (Bt-176) in raw materials and in processed food fractions.
A sample positive to a screening test needs to be further characterised to confirm its GM origin and to reveal the identity and number of the GMO(s) present. GM specific PCR-based tests can be grouped into categories (Figure 3) according to their level of specificity that depends upon the target of the DNA fragment that is amplified in the PCR (Holst-Jensen *et al*., 2003). These categories are: 'gene-specific', 'construct-specific' and 'event-specific' targeting

respectively the inserted gene, the specific construct or, as indicated before, the junction between the recipient genome and the inserted DNA at the integration locus. During the past few years various methods targeting the several GM events used for commercial purposes and falling into the different categories, were developed and validated in the EU and worldwide. The availability of updated information on the validated methods for the different targets of interest is fundamental for laboratories designing proper GMO analysis schemes. In this respect, updated information can be retrieved from http://biotech.jrc.it/methodsdatabase.htm where the Biotechnology and GMOs Unit of the European Commission's Institute for Health and Consumer Protection (IHCP) maintains a database specifically devoted to the diffusion of information on methods validated for the detection and quantification of GMOs.
In qualitative PCR, a fundamental analytical parameter is the sensitivity of a method, also known as the limit of detection (LOD), i.e. the lowest amount or concentration of analyte in a sample that can be reliably detected. Each method has its own LOD and, depending upon the purpose of the analysis, LOD requirements may vary. Indeed, with the introduction of labelling requirements in the EU (Regulation (EC) 258/97, Regulation (EC) 1139/98, Regulation (EC) 49/2000, and Regulation (EC) 1829/2003), methods are required to detect the presence of GMO in materials designated for consumption with sufficient sensitivity to ensure fulfilling of legislative requirements, that is the LOD should be less than 1/20th of the target concentration.
Several published articles have compared approaches using different primers and/or different methodologies. A review of the information indicates that no general statement on the sensitivity (LOD) can be made. Many factors, such as the intrinsic characteristics of the target sequence chosen as template, DNA quality, reagents used, etc. affect the sensitivity of the test. In general, from a practical point of view, PCR methods should detect the presence of the analyte at the LOD in at least 95 % of the cases, ensuring 5 % false negative results (ENGL, 2005). For example: for a 0.9 % nominal value the LOD should be < 0.045 % or, for 500 copies, LOD < 25 copies. For additional details and discussion on LOD definition and calculation see Holst-Jensen *et al*. (2003).

3.2.1.2. Quantification

To fulfil legal requirements (EC Regulation 1829/2003 establishing the labelling of products consisting of or containing one or more GM authorised lines in proportion higher than 0.9% calculated at the ingredient level), any product found positive needs to be quantified. Polymerase chain reaction (PCR) technology is the most widely used and reliable analytical approach for this purpose.
The basic principle of PCR is the exponential amplification of a specific

DNA fragment. Theoretically, during PCR the amount of target template doubles at each cycle leading, after n cycles, to the accumulation of 2^n molecules from each initial target sequence. As indicated above, in the typical experimental curve representing the progression of a PCR reaction over time, the exponential phase, characterised by the exponential accumulation of DNA molecules, is followed by a decrease of the amplification rate, leading to a characteristic plateau (Figure 5). In practice, PCR reactions never reach the theoretical 100% doubling efficiency and the expected number of DNA molecules (N) after n cycles is conditional to the initial number of DNA template molecules (N0) and to the reaction efficiency E, which varies between 0 and 1.

$$N = N_0 (1+E)^n$$

Several factors, such as the characteristics of the target DNA, the level of optimisation of the reaction, and other intrinsic kinetic factors affect the efficiency of a PCR reaction, leading to the characteristic loss of proportionality between the quantity of initial DNA template and final PCR product towards the end of the reaction. To overcome the limitation of conventional end-point PCR, other PCR-based techniques were developed in order to establish a more reliable relationship between the concentration of initial target DNA and the amount of PCR product.

Among the different approaches developed (Ahmed, 2002), real-time PCR was the most successful and it is currently the most accurate and powerful technique for nucleic acid quantification. Real-time PCR is a PCR system in which the amplification reaction can be monitored as it occurs, in real-time. The principal detection strategies or 'chemistries' developed for real-time PCR analysis can be divided into two main categories: Intercalating dyes (e.g. Ethidium bromide and SYBR® Green) and hybridisation probes (e.g. TaqMan®

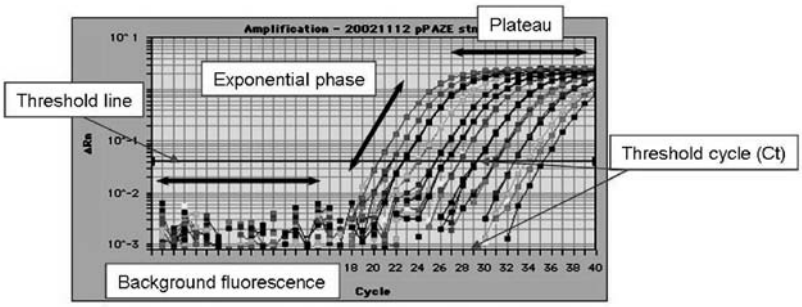

Figure 5. *Typical experimental curve representing the progression of a PCR reaction over time*

probes, fluorescence resonance energy transfer [FRET] probes and molecular beacons, Amplifluor® and Scorpions™). In either case, the accumulation of PCR products is directly monitored by the signal increase of a reporter molecule. The power of real-time PCR is that quantification occurs when the exponential growth of the amount of amplified DNA (Rn value) is sufficient to provide a signal significantly greater, and therefore distinguishable, from the background signal, and not when the PCR reaction reaches the plateau. This strategy significantly enhances the accuracy of quantification because of the direct correlation between the starting amount of template and the accumulated product at the stage at which the amplification starts to become exponential.

In real-time PCR the reaction parameter is the so-called threshold cycle or Ct value. The threshold cycle is the number of PCR cycles at which the fluorescence signal of the sample rises above the baseline signal or background fluorescence. Such a rise indicates the undisputable presence of a positive signal. The higher the initial amount of genomic DNA, the sooner the accumulated product is detected in the PCR process, and the lower the Ct value is: samples containing twice the template reach the Ct one cycle earlier, and samples with half the template reach the Ct one cycle later. In practice, the choice of the threshold line determining the Ct value is often up to the operator, representing one of the subjective elements in real-time PCR. In general, the threshold line should be placed above any baseline activity and within the exponential increase phase, which is linear after transformation on a logarithmic scale. More precisely, the threshold line should be placed where plots of different samples are parallel, and where replicates of each sample coincide the most (Figure 5).

Real-time PCR technology requires the construction of a standard curve prepared with a set of standard materials at a known analyte content. Both standard and unknown samples are analysed within the same experiment; the Ct values obtained from the amplification of the standards are used to build the regression standard curve on which Ct values of unknown samples are plotted (Figure 6).

There are various approaches to construct standard curves and to quantify GM material. In all cases, quantification is based on the independent or simultaneous amplification of two targets on the same sample: the transgenic (GM) sequence and the endogenous species-specific (e.g. maize or soy) sequence, the ratio of which is used as an estimate of the amount of GMO in a given sample. In particular, two separate standard curves are prepared for the quantification of GMOs: one for the transgene and one for the endogenous gene. The quantification of unknown samples is carried out by plotting the values of the GM and of the endogenous gene on the corresponding standard curves. The percentage of transgenic

material is then determined as being the ratio of transgenic to total target species DNA. Depending upon the chemistry, the apparatus used, and the method chosen for the real-time PCR assay, the amplification of the two targets (GM and reference sequences) can be performed either as "simplex" reactions in separate reaction wells or as "multiplex or duplex" reactions in the same reaction well using reporter dyes that fluoresce at different wavelengths (Querci *et al*., 2004).

According to European labelling legislative requirements (Regulation (EC) 1829/2003, Regulation (EC) 1830/2003) the amount (proportion) of each event present in a food or feed product must be determined and calculated relative to the corresponding ingredient. However, relevant European Regulations do not explicitly indicate on which basis the percentage of GMO content should be calculated and traditionally, standard curves for GMO quantification are prepared by using Certified Reference Materials (CRM) produced by the Institute for Reference Materials and Measurements (IRMM) of the EC's Joint Research Centre, in which the GM content is expressed as mass fractions (%) of

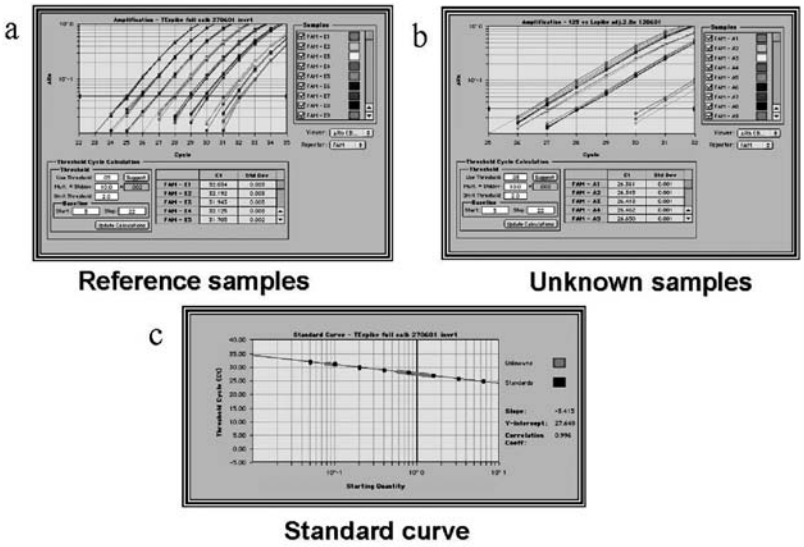

Figure 6. *Example of GMO quantification by real-time PCR. Both standard (a) and unknown samples (b) are analysed within the same experiment; the Ct values obtained from the amplification of the standards (c - black dots) are used to build the regression standard curve on which Ct values of unknown samples are plotted (c – gray dots)*

GM flour in non-GM flour (IRMM, 2007). In this case, obtained Ct values are associated with the GM content of the corresponding standard and a weight/weight ratio is used to express the GM content as a percentage. Nevertheless, since no method to correctly translate PCR results into the commonly used weight/weight unit of measurement is available, the expression of results with respect of GMO content in different product has become more and more confusing. As a consequence, the need for a single and coherent unit of measurement applicable throughout the whole production and distribution chain, from planting material (seeds) to raw materials (flour), down to the commercial goods (food or feed final products) became increasingly evident.

Only recently, with the publication of Commission Recommendation 2004/787/EC providing detailed guidance on sampling procedures and criteria for proper selection and application of molecular methods for GMO testing, was a clear recommendation to harmonise the unit of measurement of GMO content in food/feed products provided. Specifically, the Recommendation introduced the concept of haploid genome equivalents and identified results of quantitative analyses expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes as the most reliable, analytically correct, and universally applicable results, suitable throughout the agricultural and food/feed production chain (Holst-Jensen *et al*., 2006).

GMO content in a sample can be expressed as the percent of GM target DNA versus the total species DNA. In the calculation, the amount of DNA engaged in the PCR reaction is converted into copy number equivalents by considering the size of the haploid genome, or 'C value' (expressed as weight/haploid genome) of the plant species under investigation (Arumuganathan and Earle, 1991). For the determination of GM percentage in unknown samples, a calibration curve for each of the two components of the assay (GMO target sequence and reference gene sequence) is then produced by plotting the Ct values against the logarithm of the target copy number for the calibration points. The copy numbers measured for the unknown samples are obtained by interpolation from the standard curves. The GM percentage is finally calculated according to the following formula:

$$\% \text{ GM} = [(\text{copy number GM}/\text{copy number target species}) \times 100]$$

Quantitative PCR methods for GM analysis should demonstrate their suitability and relevance for the specific purpose. In the case of GMO testing for regulatory compliance, they should have the ability to provide reliable quantitative information taking into account the threshold relevant for legislative requirements. Therefore the limit of quantification (LOQ), defined as the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy, should be less than $1/10^{th}$ of the value of the target concentration with an RSDr 25% (ENGL, 2005).

3.2.1.3. DNA quality

Since all of the DNA-based procedures detailed above begin with the extraction and the purification of the DNA, a few words should be spent on DNA quality. The quality of the DNA extracted from a sample under study is of fundamental importance as it affects the performance of any downstream analytical method (Terry *et al.*, 2002). The main determinants of "DNA quality" are DNA integrity and its purity. DNA integrity can be estimated by gel electrophoresis, and it is visualised as the average length of the DNA fragments present in the sample. It is essential that the average size of the DNA fragments is larger than the transgene, or at least larger than the target sequence selected in the assay (amplicon length).

DNA extraction from raw plant materials (grains, seeds, leaves, fruits) is relatively simple to execute and generally yields DNA of good average size, suitable for PCR. On the contrary, when the DNA source is a 'matrix' consisting of food products of different origins and possibly at different stages of processing, which is the principal responsible for DNA degradation, DNA extraction becomes more complex. Nevertheless, specific procedures allowing the recovery of a sufficient amount of DNA at a purity level suitable for the analysis are available for these cases and should be applied (Zimmermann *et al.*, 1998), The second determinant of "DNA quality" is purity. In practice, "DNA purity" can be translated into DNA amplifiability: the higher the purity, the more efficient the amplification. The purity of DNA is affected by the co-purification of other molecules (e.g. polyphenols and polysaccharides) that might interfere with the amplification and therefore act as inhibitors during the PCR. Recently several studies have been published showing the effect that DNA purity and degradation rate have on both the qualitative and quantitative analysis of GMOs, in particular in processed foodstuff (Peano *et al.*, 2004; Moreano *et al.*, 2005).

3.2.2. Protein-based analytical methods

The immunological detection of novel proteins synthesised by genes introduced during transformation constitutes an alternative approach for the identification of genetically modified plants. Its application is however somewhat limited because genetic modification does not always result in the production of a new protein and, when it does, the expression level of the new protein may not be sufficiently high to be detected (Longstaff *et al.*, 1995). In addition, certain proteins may be differentially expressed in different tissues, or in different phases of the physiological development (Van der Hoeven *et al.*, 1994). The time, location and expression level of proteins are controlled by specific DNA sequences – the promoters – which can be grouped into different classes: constitutive promoters (e.g. CaMV 35S, Ubiquitin, nopaline synthase [nos]) resulting in constant ubiquitous expression; tissue specific promoters (e.g.

tuber-specific patatin, maize-derived pollen-specific CDPK and green tissue-specific PEPC) resulting in differential expression according to the location; and inducible promoters (e.g. heat-shock, light-responsive promoter) controlling protein expression as a function of specific environmental stimuli.

The most widely used immunological detection method is the Enzyme-Linked Immunosorbent Assay (ELISA) (Clark and Adams, 1977), which relies on the specific interaction between antibodies and antigens. As in all immunological approaches, the key reagents in ELISA are the antibodies, which are soluble proteins produced by the immune system in response to infection by a foreign substance, called an "antigen". When applied to GMOs, the antigens are the newly synthesised proteins. The prerequisite for the development of ELISA and of other immunological detection methods is the availability of highly specific antibodies (either monoclonal, more specific, or polyclonal, more sensitive) directed against the new protein to be detected (Ahmed, 2002). Among the different ELISA formats, the direct sandwich ELISA method (Figure 7), in which the analyte is 'sandwiched' between the capture and the detector antibodies, is the immunoassay most used in GMO detection (Stave, 2002). The procedure includes the coating of a solid support (generally the surface of a microtiter plate) with a

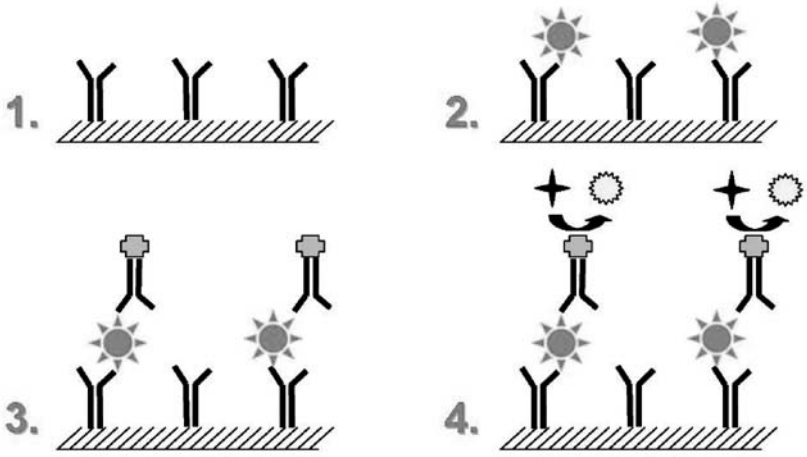

Figure 7. *Fundamental steps in the 'sandwich' ELISA test: 1) Specific 'capture' antibodies are lined at the bottom of each well of the microtiter plate; 2) upon addition of a positive sample, the 'capture' antibody binds the antigen of the target protein; 3) the 'detector' antibody, conjugated to an enzyme, is added forming a "sandwich" complex; 4) enzymatic development of a detectable signal upon addition of appropriate substrate*

specific capture antibody. When the sample of interest is added, the capture antibody binds the antigen, retaining it while unbound components of the sample are removed by washing. After washing, a second antibody, specific for a second antigenic site on the bound protein is added. The second antibody, often called the detector antibody is generally conjugated (covalently linked) to an enzyme (e.g. horseradish peroxidase) that will generate a colour signal upon addition of the appropriate substrate. The colour developed in this last phase is linearly proportional to the concentration of antigen that, itself, is directly dependant upon the amount of (GM) protein originally in the sample. Recently, an innovative variation to the standard ELISA format, called 'ELISA Reverse', based on a new conformation of the solid phase specifically designed to be immersed directly into liquid samples, was successfully applied for the simultaneous detection and quantification of CP4-EPSPS and *Cry1A(b)* proteins (Ermolli *et al*., 2006a).

The ELISA methodology has been extensively used as a tool to confirm or follow the success of plant transformation, by allowing a direct estimate of the expression level of the protein(s) synthesised by the newly introduced gene. As a consequence, information regarding the production and use of specific antibodies can be found in many articles describing the developments of transgenic plants (Mohapatra *et al*., 1999, Curtis *et al*., 1999, Padgette *et al*., 1995). However, only a few specific antibodies directed against proteins that are the products of transgenes used in approved genetically engineered crops are commercially available: among these are the antibodies against the *nptII* gene product, *NPTII* or *APH(3')II* (Wood *et al*., 1995), against the product of the *gus* gene, against some variants of the *cry* genes from *Bacillus thuringensis* (Koziel *et al*., 1993; Ermolli *et al*., 2006b) or against the *CP4-EPSPS* protein (5-enolpyruvylshikimate-3-phosphate synthase, an enzyme from *Agrobacterium* sp. strain CP4), which confers tolerance to the herbicide Roundup™ in Roundup Ready™ soybean (Padgette *et al*., 1995).

As for DNA-based approaches, only recently have protocols been developed, optimised and validated in order to allow their application for GMO testing in compliance with EU regulatory and traceability requirements. The first validation for a protein-specific ELISA method was carried out by the Joint Research Centre of the European Commission (Lipp *et al*., 2000). The method, specifically developed to target the *CP4-EPSPS* protein produced by Roundup Ready® soybean (SDI, 2003), was validated for the detection of raw materials and is therefore applicable exclusively to samples where little or no treatment has been carried out and the *CP4-EPSPS* protein is not denatured. Indeed, the use of immunoassays remains limited because of the susceptibility of proteins to denaturation. Heat treatments and industrial processing easily denature proteins, making the use of ELISA methods for processed food fractions unreliable.

A particularly interesting variation of the ELISA format are the

immunochromatographic (lateral flow) strip tests, which operate like a double antibody sandwich where the target protein is linked simultaneously by two antibodies, one monoclonal linked to a solid support, the other, a polyclonal complex, marked for identification (Stave, 2002). Compared to standard ELISA procedures, lateral flow strips have the advantage that the reaction takes place on one solid support, exploiting the protein solution flux through the absorbent strip. As a consequence, results are obtained in a few minutes and the method, although not quantitative, is very economical.

In conclusion, protein-based detection methods have strong limitations preventing their routine use for GMO testing, however they have interesting features applicable to GMO testing. In general, immunological methods are less sensitive than PCR methods and do not require the amplification of the target. As a result they are also less susceptible to 'false positives' caused by possible contaminations. Even though protein-based methods are initially time-consuming and expensive during method development and the generation of antibodies and protein standards, they are characterised by a very low per sample cost once optimised for routine use. The only unsolvable problem with protein-based methods is that they are trait-specific and cannot discriminate among different transgenic events expressing proteins with similar characteristics.

ELISA and PCR should be regarded as complementary methodologies rather than alternative to each other: the first allowing an easy, cheap initial screening, the second providing more detailed information on the quantity and type of GMO/GMOs present in a sample. As for DNA-based methods, additional information and an up-dated listing of GM-specific validated methods can be found at http://biotech.jrc.it/methodsdatabase.htm.

4. CONCLUSIONS

A number of factors must be taken into account when implementing a GMO survey in the EU in order to guarantee sufficient accuracy and compliance with legal requirements. So far, this chapter provides a comprehensive summary of all these factors: a short description of the legal system providing the regulatory framework for GMOs within Europe, an overview of the sampling protocol developed specifically for GMO testing and recommended in the EU, and a summary of the characteristics of the analytical methods currently available with their respective advantages, disadvantages and applicability features. Nevertheless, detailed descriptions of procedures and protocols, despite being necessary, are not sufficient alone to ensure harmonisation of GMO testing procedures among Member States. Instead, it is necessary to develop a frame for the standardisation of GMO testing within Europe. This process has already

begun and, during the last decade, tremendous progress has been achieved, both from a scientific and an administrative point of view. However, as pointed out earlier, not all issues have been solved and GMO testing is still evolving. A short summary of two key elements to support progress towards the harmonisation and standardisation of GMO testing is provided below, namely: the importance of method validation and the existence of international standards defining strategies to carry out validation studies.

Method validation is the process that establishes the reliability and relevance of an analytical method for a particular purpose by means of an inter-laboratory study, also known as a collaborative study or ring trial, during which a method is tested in several different laboratories to assess its performance, reliability and transferability (see Thompson *et al.*, 2002 proposing an alternative for single laboratory validation). Method validation is carried out at the end of a long and costly process, involving the development of the new method and its optimisation. During the last few years the importance of method validation for GMO testing has steadily increased because it became a mandatory pre-requisite for the acceptance of a specific product on the EU market (Regulation (EC) 1829/2003 and Commission Regulation (EC) 641/2004). Consequently, it is essential that approaches for the evaluation of method performance among different Member States and Competent Authorities are harmonised and standardised as much as possible.

Several international protocols defining criteria for the organisation of ring trials are available and routinely adopted. The International Standard Organization (ISO, 1994) and the International Union of Pure and Applied Chemistry (Pocklington, 1990) provide comprehensive standards describing various procedures to assess analytical methods performance. The Association of Official Analytical Chemists International (AOAC International, http://www.aoac.org) provides specific guidance for the validation of analytical methods. In the area of food quality and safety, the Codex Alimentarius Commission (FAO-WHO, 2005) requires the availability of specific performance information in order to include a method in the Codex commodity standard.

According to all these internationally standardised procedures, of which the examples mentioned above are only an extract, validation studies should provide detailed information regarding both the conditions of the method's applicability and the estimates for a series of indices (e.g. accuracy, precision, repeatability, reproducibility, bias) necessary to assess overall method performance. Although detailed descriptions of these indices are available in the literature, unfortunately their definitions are not consistent among different organisations. In order to facilitate the validation of methods for GMO testing and to support their standardisation within Europe, the European Network of GMO Laboratories (ENGL, http://biotech.jrc.it/) provides definitions which were agreed by taking

into consideration the specific needs of GMO testing with respect to EU legislative requirements. ENGL definitions are also adopted by the Community Reference Laboratory (CRL-GMFF, http://biotech.jrc.it/) when assessing GMO methods performance with respect to EU legislative requirements.

In addition to providing definitions of indices suitable for GMO testing, ENGL also proposes a novel approach to the assessment of method performances (ENGL, 2005). Specifically, ENGL defines two sets of criteria that any method needs to satisfy: those that need to be met by a method in order to undergo a validation study, which are referred to as 'method acceptance criteria', and those that need to be met by a method at the end of the ring trial to be considered as fit for its purpose, which are referred to as 'method performance requirements'. The advantage of this strategy is double-fold: it ensures that only 'good' methods are tested in ring trials avoiding wasting time and financial resources on methods that are not satisfactory, whilst it also ensures that all methods considered as fit for the purpose have been evaluated with respect to a priori objectively defined criteria.

As indicated earlier, detailed descriptions of procedures and protocols are necessary but not sufficient to ensure harmonisation of GMO testing procedures. Clearly, effective actions to develop a frame for the standardisation of GMO testing within Europe have been, and continue to be, taken to support the harmonisation of approaches. However, these are still not sufficient to ensure harmonisation of GMO testing. Laboratories carrying out their daily work are, ultimately, the ones providing the results necessary to assess GMO content of all market products. Consequently, in the light of effective procedures, precise protocols and optimal validation strategies, GMO testing can provide accurate results if, and only if, laboratories operate under quality assurance conditions and implement all the necessary precautions to maximize accuracy of their results. In this context, the European Committee for Standardization (CEN), in collaboration with the International Standard Organization (ISO), has developed a general guidance (ISO 24276; ISO, 2006) describing the general requirements and practical procedures for the detection of GMOs in food products.

Finally, it is hoped that this review will help in the understanding of the complexity of GMO testing. Briefly described are the technical details from sampling to final quantification, the legal frame defining GMO testing and setting specific requirements and thresholds in Europe, the actions taken to support harmonisation and to ensure maximum consistency of results among different laboratories, and finally it is underlined how crucial the role of analysts in their daily work is in ensuring the accuracy of results. The procedure of GMO testing is a long chain comprising many steps: the final result will be reliable and precise only if all the steps are treated with equal attention and scientific precision.

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