REVIEW OF GMO DETECTION AND QUANTIFICATION TECHNIQUES

European Commission, Joint Research Centre,

Institute for Health and Consumer Protection,

Food Products and Consumer Goods Unit, I-21020 Ispra, Italy

Authors: Bonfini Laura, Heinze Petra, Kay Simon, Van den Eede Guy

Date: 21/04/01

Version: final version

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1 ABSTRACT

The need to monitor and verify the presence and the amount of GMOs in agricultural crops and in products derived thereof has generated a demand for analytical methods capable of detecting, identifying and quantifying either the DNA introduced or the protein(s) expressed in transgenic plants, because these components are considered as the fundamental constituents. Several laboratories have developed therefore, methods either based on DNA detection using the polymerase chain reaction (PCR) technique, or on protein detection using enzyme linked immunosorbent assays (ELISA). Although much progress has been achieved in the development of genetic analysis methods, such as those based on the use of polymerase chain reaction (PCR), several other analytical technologies that can provide solutions to current technical issues in GMO analysis are emerging. Those includes, mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices, DNA chip technology and nanoscale GMO analysis. The development and application of reliable detection and quantitative analytical methods is essentials for the implementation of labelling rules. Appropriate sampling strategies and methods for sample preparation, as well as methods development and application to detect GMOs and their derivatives must then be followed by the establishment of validation criteria and performance assessment. Since reference samples are an indispensable part of any analytical protocol, criteria for manufacturing standard reference materials must also be considered. In order to create confidence in the testing procedures and complement enforcement requirements, there is an urgent need for using methods that are validated and officially recognised at an international level. To date, several methods for different food matrices have been submitted to validation trials. Although encouraging, the resulting reports have presented rather limited statistical analysis, which suggests a more precautionary approach in their evaluation. Indeed, the methods subjected to ring trials varied significantly in their reliability, robustness and reproducibility. Even though claims of very high sensitivity were sometimes made, they were often not supported by appropriate statistical analysis and detailed performance studies. More validation studies, especially on the quantitative PCR methods, need to be performed to provide new extraction methods for food matrices and novel GMO constructs. Considerable work remain to be done, therefore, in response to the increasing demands of consumers and policy makers to give confidence that food products on the market meet the requirements of the rapidly evolving EU legislation.

2 INTRODUCTION

Genetically modified organisms (GMOs) entered the European food market in 1996. The first product to appear on UK supermarket shelves was a genetically modified tomato puree. This product was clearly labelled and therefore anticipated the European Commission's Novel Food Regulation (EC) No 258/97 (European Commission, 1997) established in 1997, under which products containing GMOs must be labelled if they differ substantially from their conventional counterpart, either by composition, nutritional value or nutritional effects for the intended use of the food. Since two other products - Round-up Ready ® soybeans and BT-176 maize - were already authorised for marketing within Europe before the Novel Foods Regulation came into force, a specific labelling regulation was established in 1998 (EC) No 1139/98 (European Commission, 1998). This regulation requires labelling if transgenic DNA or newly expressed proteins can be found. For this purpose, qualitative methods for detection of GMOs are required. It is likewise important to investigate whether the GMO found is authorised or not; consequently, specific methods for identification of GMOs are needed.

The labelling regulation was amended two years later by fixing a threshold of 1 % for adventitious contamination of GM-material in a non-GM background (EC) No 49/2000 (European Commission, 2000). In order to determine whether a food product (on an ingredient level) contains more than 1 % GMOs (for which labelling becomes mandatory) quantitative analytical methods are necessary.

Figure 1 gives a general outline of the analytical steps that are required to comply with the current European Union GMO legislation. In general the procedure consists of three distinct steps: detection, identification and quantification.

1) Detection (screening of GMOs). The objective is to determine if a product contains a GMO or not. For this purpose, a screening method can be used. The result is a positive/negative statement. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results in all control laboratories, which can be achieved through inter-laboratory validation.

2) Identification. The purpose of identification is to reveal how many different GMOs are present and if they are authorized or not. Specific information (i.e. details on the molecular make-up of the GMOs) has to be available for the identification of GMOs. A molecular register containing - besides the scientific data - the tools for control authorities to design appropriate identification methods are being developed by the Joint Research Centre.

3) Quantification. If a food product has been shown to contain (one or more) authorised GMOs, then it become necessary to assess compliance with the 1% threshold regulation by the determination of the amount of each of the GMOs present in the individual ingredients from which it has been prepared (e.g. the maize flour). Some laboratories have claimed that it would be possible to reliably quantify GMOs at a limit of quantification (LOQ) as low as 0.1%. However, these claims have never been confirmed with appropriate validation studies.

The need to monitor and verify the presence and the amount of GMOs in agricultural crops and in products derived thereof has generated a demand for analytical methods capable of detecting, identifying and quantifying either the DNA introduced or the protein(s) expressed in transgenic plants, because these components are considered as the fundamental constituents (reviewed in Bindler et al., 1999; Gachet et al., 1999; Gadani et al., 2000; Hemmer, 1997; Lüthy, 1999; Meyer, 1999; Messens et al., 1999; ILSI Europe Report, 2001).

Consequently, several laboratories have developed methods either based on DNA detection using the polymerase chain reaction (PCR) technique, or based on protein detection using enzyme linked immunosorbent assays (ELISA) (Anklam et al., 2002). However, the methods vary in their reliability, robustness and reproducibility; in combination with different levels of cost, complexity, and speed etc. Moreover, there is no one method that is applicable to all circumstances. A further consideration is the claim of very high sensitivity reached in the analysis even in absence of clearly proven detailed performance studies.

In order to obtain comparable results, and hence giving consumers and producers confidence in the testing methods, there is an urgent need for internationally validated methods, which could serve as reference methods. To date, several methods have been validated at a European level, and the trend for such studies is increasing at an international level.

Nevertheless, in order to be able to demonstrate that the results from the use of a validated method are correct, control laboratories should participate in proficiency testing, which is also a valuable step in the preparation for accreditation. A series of first proficiency tests regarding GMO analysis in food has been launched recently in Europe.

This review summarises in the section “State of the art in detection and quantification techniques”, the status of the GMO analysis technologies together with new areas of analytical investigation. In the section named “A need for harmonized methodology” this review covers the needs and procedures for harmonized methodologies in GMO analysis.

3 STATE OF THE ART IN DETECTION AND QUANTIFICATION TECHNIQUES

The assays on raw materials are generally carried out with the polymerase chain reaction (PCR method) or with immunological assays such as the enzyme-linked immunosorbent assay (ELISA). An immunological assay is based on the specific binding between a protein and an antibody and therefore, any conformational changes in the tertiary structure of the protein render the test ineffective. Such conformational changes are induced frequently during food processing and hence, processed foods are generally analysed with the PCR method. Although much progress has been achieved in the development of genetic analysis methods, such as those based on the use of polymerase chain reaction (PCR), several other analytical technologies that can provide solutions to current technical issues in GMO analysis are emerging. Those includes, mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices, DNA chip technology and nanoscale GMO analysis. Irrespectively of a variety of potentially available methods for DNA analysis, so far only PCR in its different formats has found broad application in GMO detection/analysis as a generally accepted method for regulatory compliance. This Part of the review covers in detail the principle and the application constrains of the PCR method when is used as analytical tool in GMOs detection, identification and quantification. In the following chapters it discusses the principles and applications of immunological assays and of the emerging analytical technologies.

3.1 DNA ANALYSIS METHODS

As DNA is a rather stable molecule it is the preferred analyte for almost any kind of sample (raw materials, ingredients, processed foods). In addition, PCR, which is the most common DNA detection method, is very sensitive and therefore, very small aliquots of vegetal material are required for the analysis. Provided that the laboratory sample is representative for the field sample and that it has been adequately homogenised (see chapter 4.1) aliquots between 100 mg (Pietsch et al., 1997) and 350 mg (Zimmermann et al., 1998a) are adequate for DNA extraction procedures in the laboratory.

3.1.1 DNA EXTRACTION METHODS

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

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

i) Breakage of cell walls, which is usually achieved by grinding the tissue in dry ice or liquid nitrogen;

ii) Disruption of cell membranes by a detergent (e.g. CTAB or SDS) which is (besides EDTA and a buffering salt like Tris-HCl) a necessary component of any DNA extraction buffer;

iiia) Inactivation of endogenous nucleases by the addition of detergents and EDTA, a chelator of Mg2+, which is an obligatory co-factor of many enzymes;

iiib) Addition of proteinase K for inactivation and degradation of proteins, particularly in protocols using DNA-binding silica columns;

iva) Separation of inhibitory polysaccharides from DNA through differential solubilisation in solutions containing CTAB;

va) Separation of hydrophobic cell constituents from DNA, e.g. lipids and polyphenols by extraction with an organic solvent like chloroform;

via) Separation from the detergent and concentration of DNA by alcohol/salt precipitation.

ivb-vib) Alternatively, the separation of DNA from other cell components can be achieved via purification on a DNA-binding silica column (Spoth and Strauss, 1999; Melzak et al., 1996).

Currently, three different approaches of DNA isolation from plant material and plant-derived products are favoured for GMO detection: the CTAB-method, the DNA-binding silica column method (various commercially available kits), or a combination of the two. Even though the yields are low, the quality and purity of the DNA is satisfactory higher than with other procedures e.g. Alkali, Chelex100, and ROSE methods (Zimmermann et al., 1998a).

The CTAB method has been originally described by Murray and Thompson (1980). They were able to extract from plants pure DNA of high molecular weight. The procedure roughly follows the above-mentioned outline (1a-6a), using CTAB as a detergent in the DNA extraction buffer. It appears to be an efficient method for a wide range of plant materials and plant-derived foods and it provides a good separation of DNA from polysaccharides. This procedure is part of the official protocols for GMO detection according to the German Food Act LMBG §35 (L24.01-1, 1997; L23.01.22-1, 1998; L25.03.01, 1999).

DNA-binding silica columns have proven to be suitable for extraction of good quality DNA, and the use of one of the commercially available kits is described in the official Swiss method for GMO detection (Schweizerisches Lebensmittelbuch, 1998). However, it has been reported that polysaccharides tend to bind to silica columns affecting the efficiency of the separation.

Extraction of DNA from complex matrixes or food products is not always successful. Failures in extracting detectable DNA levels have so far been reported for soybean sauce, refined soybean oil (Meyer and Jaccaud, 1997; Pauli et al., 1998), and distilled ethanol produced from GM potatoes (Hupfer et al., 1999b). However, method optimisation has allowed Hellebrand et al., (1998) to extract DNA from cold pressed as well as from refined rapeseed oil, although the DNA could not be identified unequivocally. Pauli and co-workers were able to extract detectable DNA levels from a large variety of food products and processing stages, but did not succeed in extracting it from refined sugar and oil (Pauli et al., 2000).

It should be stressed that OD 260 measurements, generally used to determine DNA concentration in extract solutions are not sufficient when products are processed and accurate quantitative information is needed.

3.1.2 PCR DETECTION

3.1.2.1 Principle

The PCR reaction allows the million-fold amplification of a specific target DNA fragment framed by two primers (= synthetic oligonucleotides, complementary to either one of the two strands of the target sequence). In principle, the PCR is a multiple-process with consecutive cycles of three different temperatures, where the number of target sequences grows exponentially according to the number of cycles. In each cycle the three temperatures correspond to three different steps in the reaction.

In the first step, the template, i.e. the DNA serving as master-copy for the DNA polymerase is separated into single strands by heat denaturation at ~94°C.

In the second step, the reaction mix is cooled down to a temperature of 50-65°C (depending on the GC-content) to allow the annealing of the primers to the target sequence. Primer hybridisation is favoured over DNA-DNA hybridisation because of the excess of primers molecules. However, the annealing process is uncontrolled and can give rise to a large number of mismatched DNA duplexes.

In the third step, the annealed primers are extended usually by a *Thermus aquaticus* (Taq) polymerase at the optimum temperature of 72°C. With the elongation of the primers, a copy of the target sequence is generated.

The cycle is then repeated 20 to 50 times, depending on the amount of DNA present and the length of the amplicon (= amplified DNA fragment).

Since the development of the PCR technique new thermophilic polymerases have been placed on the market (e.g. Pfu, Pwo, Tma, Tli, Tfl). Vent™ -, DeepVent™-, Pfu, and UITma™- DNA polymerases have a 3'- 5' exonuclease activity that allows the removal of mismatched residues until a correctly base-paired terminus is generated. However, the 3'- 5' exonuclease activity can cause degradation of the primers. Therefore, the enzyme should only be added after the reaction has started, or in alternative, chemically modified primers should be used. Their proofreading activity is of great value for genetic engineering applications or other specialised experimental procedures, yet is counterproductive for screening or quantitative GMO determinations. Both *Taq*, and Ampli*Taq* (the genetically recombinant version of the enzyme) DNA polymerases only possess a 5' to 3' exonuclease activity, which removes nucleotides ahead of the growing chain, and therefore remain the enzymes of choice for GMO analysis. Table 2 lists the properties of some thermostable DNA polymerases currently in use for PCR applications (Newton and Graham, 1994).

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

Besides the well-known points of consideration for the primer selection (e.g. no inverted repeats within one primer, no complementarity of one primer to the other, presence of a GC-rich 3'end, etc.), the choice will depend very much on the objective of the PCR analysis.

For routine screening purposes one should focus on target sequences that are characteristic for the group to be screened. Genetic control elements such as the cauliflower mosaic virus 35S promoter (P-35S) and the *Agrobacterium tumefaciens* nos terminator (nos3') are present in many GMOs currently on the market (Hemmer, 1997). The first GMO screening method has been originally introduced by Swiss and German scientists (Pietsch et al., 1997) and is based on the detection of the P-35S and nos3' genetic elements. However, a few approved GMOs are not screenable/detectable with the P-35S or the nos3' primers and additional target sequences are needed to guarantee a complete screening procedure. We list in Table 3 the genetic elements introduced in 28 approved GM products (as matters of stand in 1997). Hemmer (1997) proposed them as generic GMO markers for the design of appropriate screening strategies. A further aspect is the choice of primers that allow to detect as many variants as possible of a GMO marker, e.g. there are at least 8 variants of P-35S used in GM crops (Hemmer, 1997). It should be stressed however, that the detection of these generic GMO markers it only indicates that the analysed sample contains DNA from a GM plant, but does not provide information on the specific trait that has been engineered in the plant.

3.1.2.2 Confirmatory assays

Confirmation/verification of the identity of the amplicon is necessary to assure that the amplified DNA is really corresponding to the chosen target sequence and is not a by-product of un-specific binding of the primers. Several methods are available for this purpose:

Gel electrophoresis is the simplest approach to control if the PCR products have the expected size. However, there is a risk that an artefact having the same size of the target sequence may have been amplified. Therefore, the PCR products should additionally be verified for their restriction endonuclease profile (Meyer, 1995; Pietsch et al., 1997).

Even more reliable is a Southern blot assay, whereby the amplicon is separated by gel electrophoresis, transferred onto a membrane and hybridised to a specific DNA probe (§35 LMBG L24.01-1, 1997; LMBG L23.01.22-1, 1998; LMBG L25.03.01, 1999; Hupfer et al., 1997).

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

The most reliable way to confirm the authenticity of a PCR product is its sequencing. However, only few laboratories are equipped to carry it out this approach routinely. Hence, only a few authors have reported its use (Ehlers et al., 1997; DMIF-GEN Final Report, 1999; Hupfer et al., 1997).

3.1.3 LIMIT OF DETECTION (LOD)

The efficiency of the PCR - as with any other DNA assay - depends on DNA quality and purity:

DNA quality is determined by fragment length and degree of damage. This may be caused by exposure to heat, low pH, and/or nucleases that cause hydrolysis, depurination and/or enzymatic degradation. Therefore, DNA quality varies according to the material under examination, the degree of processing the sample has been subjected to, and the DNA extraction method applied. DNA isolated from processed foods and certain agricultural matrixes is usually of low quality, and available target sequences may be rather short, e.g. 100-400 bp for soybean protein preparations and processed tomato products (Hemmer, 1997). Thus, an appropriate choice of primers to obtain short amplicons should be made.

DNA purity can be severely affected by various contaminants in food matrices (Vroh Bi et al., 1996). Contaminants may be substances that originate from the material under examination, e.g. polysaccharides, lipids, and polyphenols (Rogers and Bendich, 1988; Gadani et al., 2000), or chemicals used during the DNA extraction procedure, as reported for CTAB - (cetyltrimethylammonium bromide or hexadecyltrimethyl ammonium bromide), ROSE and the Alkali method (Zimmermann et al., 1998a). For example, the Taq polymerase, the key enzyme used in the PCR reaction is inhibited by polysaccharides, ethylenediaminetetraacetic acid (EDTA), phenol, sodium dodecylsulfate (SDS), etc.

Jankiewicz et al. (1999) estimated for their PCR experiments the theoretical detection limit to be 0.005% GMO/non-GMO (w/w), corresponding to 30 copies of herbicide tolerant soybean haploid genome copies or to 9 copies of insect resistant maize haploid genome. The authors showed that the practical detection limit is significantly (20x) lower; i.e. 0.1% GMO/non-GMO (w/w) corresponding to 596 copies of RR soybean haploid genome or to 185 copies of Bt maize haploid genome. No

claims concerning limit of detection have been supported by appropriate validation studies. In addition, the accuracy of the method is certainly dependent on the type of GMO, food matrix and processing involved. Finally, the analyses of samples containing a low concentration of GMOs may even show a greater error.

It might be advisable to analyse the food chain from the very beginning (e.g. raw materials or grains) for GMOs thus facilitating the situation in which one crop type might be processed into a large variety of different products, some of which being used as ingredients.

3.1.4 PCR METHODS FOR GMO IDENTIFICATION

For unequivocal identification, primer selection has to be based on target sequences that are characteristic for the individual transgenic organism, e.g. the cross-border regions between integration site and transformed genetic element of a specific GMO (the so-called edge fragments), or specific sequence alterations due to truncated gene versions (i.e. coda, or altered codon usage). For example, a combination of screening and gene specific detection methods has been used on *Solanaceous* crops to detect the "universal" GMO markers P-35S and nos3' and widely used virus resistance genes (Jaccaud et al., 2000; Gadani, 2000).

In a recent extensive review of GMO detection methods applied to tobacco, the Task Force Genetically Modified Tobacco – Detection Methods of CORESTA (Cooperation Centre for Scientific Research relative to Tobacco, Paris, France) (Bindler et al., 1999) has reported on the successful application of the above-mentioned PCR strategy to identify different types of dry GMO tobacco leaves, and has highlighted the advantages and drawbacks of confirmatory tests such as nested PCR, restriction enzyme digestion of amplicons, and nucleotide sequencing of the detected genetic elements.

Generally one should try to avoid target sequences that may occur as natural contaminants in the sample, i.e. DNA from plant viruses and bacteria, because of the risk of false-positives. Therefore, a sample with a positive signal in P-35S/nos3' screening should be analysed for naturally occurring CaMV and *A. tumefaciens* infection, respectively (Wolf et al., 2000). However, it should be considered that the host range of the cauliflower mosaic virus is restricted to *Cruciferous* plants such as oilseed rape, and that the nos3' terminator sequence is found only in certain strains of *A. tumefaciens*, which are pathogenic to certain crop species. As to the *A. tumefaciens* frequently found in soil, it is generally not virulent, i.e., it does not carry the Ti-plasmid with the T-DNA and the oncogenes. Thus the nos3' gene and its control elements are not present in this naturally occurring strain.

Most methods so far available for GMO screening and identification are listed in Table 4A, 4B, and 4C. If available, the reference where the original target sequence has been published is also included. Whereas in the past scientist have been focusing on the development of screening methods, recently more emphasis has been given to the development of identification methods. P-35S, nos3' and nptII have been widely used as GMO marker for screening purposes, whereas for identification purposes cross border sequences have been usually utilised.

Only a continuous survey of all data available on GMOs - especially the introduced genetic elements and their integration sites, not only for GM products approved for market release but also for any other GMO released for field trials worldwide – can guarantee a complete/comprehensive monitoring/detection of GMOs. Within the European Union preliminary efforts towards the establishment of a database providing at least part of the relevant information have already been made (DMIF-GEN Final Report, 1999). These efforts will be continued and extended within the European Commission Joint Research Centre (JRC) project "Development of a GMO register

consisting of a database and accompanying bioinformatic tools designed for monitoring purposes as required under Directive 90/220/EEC" (Van den Eede, 2000).

3.1.5 PCR METHODS FOR GMO QUANTIFICATION

A major drawback of the conventional PCR is lack of accurate quantitative information due to the effect of the amplification efficiency (E). If the reaction efficiency for each amplification cycle would remain constant, the concentration of DNA following PCR would be directly proportional to the amount of initial DNA target. Unfortunately, E is not a constant parameter but varies between different reactions, as well as within the same reaction, particularly in the later cycles of the PCR, when products are formed with an unknown reaction rate and in a non-logarithmic fashion. In order to have the maximal sensitivity, product formation is measured when the amplification reaches the maximum product yield (known as the plateau phase), i.e. when the correlation between the product concentration and the number of the initial target molecules is very poor (Bindler et al., 1999; Gadani et al., 2000; Köhler et al., 1996; Raeymaekers, 1993; Rasmussen, 1994). Thus, conventional PCR relies on end-point measurements, when often the reaction has gone beyond the exponential phase because of limiting reagents. Other PCR-based techniques, such as quantitative competitive PCR (QC-PCR) and real-time PCR have been recently developed which address the problem of establishing a relationship between the concentration of target DNA and the amount of PCR product generated by the amplification.

For relative GMO concentrations in food mixtures, the quantification of a GM-marker has to be normalized to a plant-specific reference gene (Wurz, et al., 1999; Hübner et al., 1999a; Hupfer et al., 2000). In practice, accurate relative quantification might be achieved by a combination of two absolute quantification reactions: one for the GMO-specific gene and a second for the plant reference gene. With the assumption that GMO material has been submitted to the same treatment as the non-GMO material, the measurement can be expressed as genome/genome% (g/g %) or weight/weight (w/w%).

For what concern the quantification of a GMO-specific gene in a mixture, such as in quantitative GMO analysis, there is currently an intense debate on how the GMO concentration should be expressed. At present, the argument is somewhat academic, since control laboratories first measure the DNA concentration in the sample, and then for equal amounts of DNA they measure the number of copies that are found for a GMO-specific sequence and for a crop-specific sequence. The ratio of both, taking into consideration the respective number of copies per genome, gives the percentage of the GMO present. Opponents of this approach argue that it is very difficult to quantify DNA and that in addition, such a methodology can only give relative data. Currently however, there is no real valid proposal for substituting the genome/genome ratio with some other, more manageable units of measurement. Often, the genome/genome ratio is mentioned to be equal to the DNA weight/weight ratio. This assumption is probably an oversimplification since the genome size of crops is not a constant value and since there might be a variation of up to 25% within individual plant varieties.

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

i) Co-amplification of a target analyte with an internal standard to correct for the decrease in reaction efficiency, such as in quantitative competitive PCR (QC-PCR) and in double QC-PCR.

ii) Measurement of the PCR amplicon at an early stage of the reaction, when the efficiency is still constant and the product concentration still correlates well with the concentration of the initial target molecules, such as in PCR-ELISA and real-time PCR.

The PCR procedures belonging to these two analytical strategies are discussed here in more detail.

3.1.5.1 QC-PCR

QC-PCR involves the co-amplification of unknown amounts of a specific gene target and of known amounts of an internal control template in the same reaction tube by the identical primer pair. The control template is typically a deletion product of the target sequence so that identical primers and reaction conditions can be maintained to generate amplification products that should not differ, however, by more than 40 bp. Multiple PCR reactions are needed as each sample is amplified with increasing amounts of competitor, while maintaining constant the sample volume/concentration. Quantification is achieved by comparing the equivalence point at which the amplicon from the competitor gives the same signal intensity of the target DNA on stained agarose gels (Gilliland et al., 1990). The procedure assumes that the amplification reactions of the target sequence and of the internal standard (i.e. the competitor) proceed with the same efficiency in any phase of the reaction, including the plateau phase. It cannot be excluded however that the efficiencies of the two reactions are instead different, because, for instance, the competitor DNA is purified from plasmid DNA preparations that are of higher quality than the plant-derived target DNA preparations.

The QC-PCR method described by Studer et al. (1998) for the quantification of Roundup Ready® soybean and Maximizer® maize was successfully tested in an inter-laboratory trial with twelve European control laboratories (Hübner et al., 1999b; Hübner et al., 1999c). Hardegger et al. (1999) developed a QC-PCR screening method based on the quantification of the 35S promoter and the nos terminator. However, the fact that the copy number of these generic markers may vary between different GM–lines needs to be considered when this method is applied.

3.1.5.2 Double QC-PCR

Wurz et al. (1999) reported on the assessment of the so-called “Double QC-PCR”, a technique in which the concentration of soybean DNA in different samples is first normalized using a QC-PCR quantification of the soybean-specific lectin le1 gene. When the same samples are submitted to a second QC-PCR with a GM marker, relative quantification can be established. However, since the generation of calibration curves is rather complex and the accuracy depends on various factors, Wurz et al. (1999) have only used one competitor concentration, equivalent to 1% GM soybean (Roundup Ready®). Therefore, the method could only distinguish if a sample contained more or less GM material than the calibration concentration of 1%. Within this determination, some degree of uncertainty could not be avoided. More recently, Hupfer et al. (2000) described a Double QC-PCR method for the quantification of Bt maize, in which multiple competitor concentrations were used for the quantification of the amount of transgenic DNA, as well as for the quantification of the total amount of amplifiable maize DNA. They obtained a good correlation between the actual and measured GMO concentrations, even when the amount of amplifiable DNA was reduced by heat treatment to less than 20% of the initial value. Furthermore, they showed that the reduction of amplifiable DNA could not be observed by UV measurement, which demonstrates the need for an accurate quantification of both GM and endogenous plant markers. The use of Double QC-PCR might reduce the inter-laboratory differences observed in ring trial studies.

3.1.5.3 PCR-ELISA

PCR-ELISA uses the strategy of the second group and can be quantitative if the PCR reaction is terminated before a significant decrease in amplification efficiency occurs. PCR-ELISA has been used to quantify relatively low amounts of PCR products (González et al., 1999; Landgraf et al., 1991). Despite the fact that relative quantification using PCR-ELISA has been applied in different fields (e.g. Taoufik et al., 1998) and that a GMO detection kit using PCR-ELISA has been commercialised (D-Genos, Angers, France), this technique has not been widely adopted for accurate GMO quantification purposes.

3.1.5.4 Real-time PCR

Another strategy of the second group that improves accuracy, specificity and throughput of quantitative PCR is "Real-time PCR" (Heid et al., 1996). This technique was originally developed in 1992 by Higuchi et al. and is rapidly gaining popularity due to the introduction of several complete real-time PCR instruments and easy-to-use PCR assays. A unique feature of this PCR technique is that the amplification of the target DNA sequence can be followed during the whole reaction by indirect monitoring of the product formation. Therefore, the conventional PCR reaction has to be adapted in order to generate a constant measurable signal, whose intensity is directly related to the amount of amplified product. Real time detection strategies rely on continuous measurements of the increments in fluorescence generated during the PCR reaction. The number of PCR cycles necessary to generate a signal that is significantly and statistically above noise level is taken as a quantitative measure and is called cycle threshold (Ct) (Figure 2A). As long as the Ct value is measured at the stage of the PCR where the efficiency is still constant, the Ct value is inversely proportional to the log of the initial amount of target molecules (Figure 2B).

One of the most popular assays for real-time PCR is the Taqman® or 5'-exonuclease assay, which employs a fluorogenic probe consisting of an oligonucleotide with both a reporter and a quencher dye attached (TaqMan® probe, see Figure 3A). When the probe is intact the reporter fluorescence is quenched by the proximity of the quencher dye. Due to its target-specific sequence, the probe anneals specifically to the amplification product (target DNA) between the forward and the reverse primers. If hybridisation has occurred, the 5'-3' exonuclease activity of the Taq polymerase cleaves the internal probe during the extension step of amplification. The cleavage reduces the quenching effect and the fluorescent signal of the reporter dye becomes a measure of the amount of amplification product generated (Figure 2A). Because the development of the fluorogenic reporter signal takes place only if both the PCR primers and the TaqMan® probe anneal to the target DNA, the specificity of real time PCR detection is considerably higher than that of conventional PCR. The relative quantification of the target gene is made possible by preparing a standard curve from known quantities of an additional endogenous gene and extrapolation from the linear regression line.

Wurz et al. (1999) have described how real-time PCR is amenable to relative quantification of GM soybean. Two different quantification reactions were applied to calculate the fraction of GM soybean present in a sample: The first reaction was used for the absolute quantification of total soybean DNA while the second reaction was used for the absolute quantification of GM soybean DNA. Vaïtilingom et al. (1999) described a similar method for the quantification of Maximizer® maize and Roundup Ready® soybean and demonstrated the suitability of real-time PCR for the relative quantification of GM material in different food ingredients. The authors also reported that the quantification of the GM marker and an endogenous reference gene could be combined in a single tube by using a multiplex PCR approach. In this way, the quantitative analysis of each sample was not affected by random differences in experimental factors such as i.e. pipeting errors. In addition, it allowed the utilisation of an internal standard, which is a better control for false negative results. The use of multiplex PCR for quantitative determination is made possible by the utilisation of different reporter dyes, which can be detected separately in one reaction tube. Multiplex reactions are economical and allow accurate relative quantification without previous estimation of DNA quantity or copy numbers. With a multiplex reaction it can be established a direct correlation between results of real-time PCR and % of GMO. This reduces the variation and allows accurate data interpretation by simple statistical evaluation of the quantification results (Pijnenburg, 1998). Due to the above-mentioned advantages, multiplex real-time PCR is increasingly applied in genetic analysis (e.g., Johnson et al., 2000; von Ahsen et al., 2000). Siler et al. (2000) developed an assay similar to that described by Vaïtilingom et al. (1999), with an additional assay for the 35S promoter and the nos terminator. Höhne et al. (2000) successfully applied to GM maize a multiplex real-time PCR assay

that employed zein as the endogenous reference gene and the P-35S promoter as a GMO marker for four different types of GM corn. A detection limit of 0.01 % GM corn/non-GM corn was obtained.

In addition to the TaqMan® assay, various other techniques for the indirect monitoring of the PCR products have been recently described. The dsDNA dye SYBR® Green I stain (Molecular Probes, Eugene, OR) has been employed in place of Ethidium bromide as a double stranded DNA (dsDNA) dye in agarose gels (Higuchi et al., 1992) to reduce background and allow better real-time monitoring of product formation (Wittwer et al., 1997). This dye was successfully used for quantification over a 108-fold range (Wittwer et al., 1998), although it generated experimental artefacts due to the formation of undesirable products such as primer dimers. However, such products can generally be recognized by a melting curve analysis (Ririe et al., 1997).

Other real-time PCR techniques that instead make use of amplicon-specific probes are: fluorescence resonance energy transfer (FRET) probes (Wittwer et al., 1997), molecular beacons (Tyagi and Kramer, 1996) and Scorpion™ probes (Whitcombe et al., 1999) (Figure 3).

FRET probes (Figure 3B) give a high level of specificity due to the use of two adjacent hybridisation probes. Since FRET probes are not cleaved during PCR cycling, melting curves can be applied as a confirmatory test for the PCR (Wittwer et al., 1998; Wittor et al., 1998). FRET probes are the technology of choice for sequence-specific quantification with the LightCycler™ (Roche Molecular Biosystems, Indianapolis, IN). Since the introduction of the colour compensation feature in the LightCycler™ software, FRET can be used in a multiplex PCR format for the quantification of multiple targets in one reaction (Bernard et al., 1999; von Ahsen et al., 2000). A variant of the FRET approach has been reported by Wittwer et al. (1997) who have used a 3'-end primer labelled acceptor in combination with a 3'-end donor labelled probe, which is designed to hybridise on the opposite strand of the labelled primer. Since only one probe is used, this approach has a lower level of specificity.

Molecular beacons (Figure 3C) have been successfully employed in real-time PCR and for the generation of melting curves (McKillip and Drake, 2000; Leone et al., 1998), including the multiplex PCR format (Cayouette et al., 1999). Although molecular beacons appear to be less popular than TaqMan® and FRET probes for applications in quantitative PCR, they are widely used for discriminating single base pair differences (SNP) (Tyagi et al., 1998; Bonnet et al., 1999). Therefore they may be tailored for the detection and quantification of new GM-crops that feature single nucleotide genetic modifications (e.g. produced by techniques such as chimeraplasty).

Other advances in real-time PCR include self-probing amplicons that are generated during the PCR reaction (Whitcombe et al., 1999), with one of the PCR primers being replaced by a so-called Scorpions™ primer (Figure 3D). The unimolecular nature of the hybridisation event might have advantages over probe systems such as TaqMan®, FRET and molecular beacons. Scorpions™ can be used as an alternative technology in most of the real-time assays.

Thanks to the increasing interest in real-time PCR, new fluorescent dyes, quenchers and reporter-quencher combinations are being investigated to explore the possibilities of multiplying the number of targets which can be measured within one reaction (Nasarabadi et al., 1999)

The growing number of commercially available real-time PCR thermocyclers is an indicator of the success of this technology. At least four companies currently manufacture real-time PCR hardware. Detailed information on each instrument can be obtained by visiting the manufacturer's web site.

Besides the possibility of accurate quantification, the advantage of real-time PCR is the ability to increase the sample throughput in comparison to other quantification techniques as post-PCR

analysis is reduced to data treatment. Furthermore, with real-time PCR, the possibility of introducing variability and false positives is reduced. Since both amplification and detection are combined in one step carried out in a closed tube, the risk of cross contamination with PCR amplification products is minimized (Vaïtilingom et al., 1999).

Presently, real-time PCR can be considered as the most powerful tool for the detection and quantification of GMOs in a wide variety of agricultural and food products. While multiplex PCR formats with an endogenous reference gene will be able to increase the accuracy, precision and throughput of this technique, a more comprehensive evaluation of real-time protocols and formats (including data treatment) needs to be performed in the near future, possibly through inter-laboratory ring trials.

3.1.6 LIMIT OF QUANTITATION (LOQ)

It can be calculated that if samples are distributed in a binomial manner, 299 maize kernels are needed to detect ≤ 1% GMO with a confidence of 95% (Ryman, 1999). In other words, if the test result is negative, then there is 95% probability that the true concentration is 1% or less. However, to quantify at around this level, a simple random sample of 9801 particles is required to produce an estimate of ± 20% at a 95% confidence interval (Cochran, 1977); for example, if the true concentration is 1.2, then estimates would range from 1.0 to 1.4%, 95% of the time.

If DNA is extracted e.g. from homogenized maize chips, it might well happen that in the final reaction mixture for DNA amplification, the total genome copy number is below the critical particle size (i.e. 299 in the case of PCR for screening purposes at 1%) and hence, that the relationship between sample size at the beginning and at the end of the experiment has been lost.

These points can be better clarified by a more detailed explanation of genomic plant organisation. The DNA amount in the unreplicated, haploid nuclear genome of an organism is referred to as its C-value. *Zea mays ssp. mays*, for instance, has ten chromosomes in its haploid nuclear complement. The DNA content of the unreplicated haploid complement (n = 10) is known as the 1C value, while that of the replicated diploid complement (2n = 20) is the 4C value. DNA amounts are usually expressed in picograms (pg), or in megabase pairs of nucleotides (Mb) (Bennet et al., 2000). Significant intra-specific variation in nuclear DNA amount occurs in *Zea mays ssp. mays*, so a DNA C-value cannot be necessarily correct for all lines of this specie. Laurie and Bennett (1985) estimated that 1C total nuclear DNA values range from about 2.45 pg (2,364 Mb) to 3.35 pg (3,233 Mb). In 1994 over 120 estimates of DNA C-values for more than 100 genotypes of *Zea mays ssp. mays* from over 25 sources were known. The 1C value for wheat (*Triticum aestivum*), and soybean (*Glycine max*) are 17.352 pg (16,978 Mb) and 1.25 pg (931 Mb) respectively. Although a discussion on genome size and copy numbers must consider the state of the genome (i.e. if it is present as unreplicated diploid/triploid, or duplicated diploid/hexaploid), it is significant to include in this context the average numbers of 1C values. Based on the 1C values presented above, 100 ng of DNA (which is the typical amount used in a PCR reaction) may contain approximately $8x10^4$ copies of soybean genome, $3.8x10^4$ copies of maize genome or $5.8x10^3$ copies of wheat genome (see Table 5). This corresponds to a total of 80 genome copies for soybean, 38 genome copies for maize and 5 genome copies for wheat if only 0.1% of 100 ng of DNA is of GMO origin. Consequently, the theoretical detection limit (which is equivalent to one copy of unreplicated haploid genome) in a 100 ng DNA reaction is about 0.001%, 0.003% and 0.02% in the case of soybean, maize and wheat respectively.

Moreover, sampling requirements may demand a (mean) minimum of e.g. 35 units, and thus this detection limit is reached at much higher concentrations. This means that nature itself has set some limits of detection that can only be overcome by increasing the reaction volumes and/or quantities for analysis.

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

Other PCR applications are discussed in the following sections 3.1.7 and 3.1.8.

3.1.7 PCR METHODS FOR VARIETY FINGERPRINTING

Analysis of plant and animal genomes using DNA markers are proving valuable in breeding programs to rapidly develop improved crop and livestock strains with enhanced productivity. Genome studies are enabling scientists to gain valuable insights into how the crop and livestock genomes are organized. They are also providing a multitude of practical applications such as variety identification through DNA fingerprinting and development of genetic maps, which facilitate indirect selection of economic traits, such as disease resistance.

The four principal approaches for identifying polymorphic DNA markers have been so far: Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), micro satellites or short sequence repeats (SSR) and Amplified Fragment Length Polymorphism (AFLP). All four approaches have some merits but also a few inherent disadvantages.

The RFLP is a laborious technique that relies on Southern blotting and results in the detection of fewer alleles than other methods. The RAPD approach is instead sensitive to reaction conditions and presents problems of reproducibility. The micro satellites strategy on its turn provides high throughput data on polymorphism but requires lengthy studies involving cloning and sequencing to obtain information on flanking nucleotide sequences. The AFLP approach, which combines RFLP and RAPD techniques, has been developed by a private company (Vos et al., 1995). It involves the restriction digestion analysis of a PCR reaction, and thus is quickly becoming very popular among plant genome scientists. In respect to the previously developed techniques the AFLP approach presents many advantages: it detects more point mutations than RFLPs, it enables the identification of very large number of polymorphic DNA markers, it is simpler than the micro satellites technology and does not need prior sequence information. Even though it is highly informative, however, it requires multiple procedures, is costly, and demands the use of radioactivity. The AFLPs were initially named to rhyme with RFLPs. Subsequently it was realised that AFLP involves the detection of 'presence' or 'absence' of restriction fragments rather than differences in their lengths. AFLP have been shown to discriminate between plant varieties (Cooke, 1999; Preston et al., 1999), even in processed vegetal materials (Rossi et al., 2000).

Interestingly, AFLP has been investigated for its potential in the combined identification of variety genotypes and the monitoring of very low levels of GM material. Recent experimental findings (DMIF-GEN Final Report, 1999) indicated that the AFLP technology could be used for the detection of genetic modifications by using GMO-specific primer in conjunction with AFLP primers specific for the flanking genomic region.

3.1.8 MASS SPECTROMETRIC DETECTION OF PCR PRODUCTS

Since traditional molecular biology techniques (e.g. gel electrophoresis) often lack accuracy and possibility of high throughput automation, mass spectrometry (MS) has also been investigated as an alternative strategy for genotyping. The most promising methods are based on electro spray ionisation (ESI) and matrix-assisted laser-desorption-ionization mass spectrometry (MALDI) (Jackson et al, 2000). MALDI is coupled to a time-of-flight (TOF) analyser whereas ESI is lined to either a quadrupole or an ion trap analyser. Both techniques allow the detection of non-volatile and

thermally labile molecules that have molecular weights as low as 1-2 kDa. MALDI-TOF is normally applied for the identification of oligomers of less than 100 nucleotides (about 30 kDa), however a modified instrument, an infrared MALDI single-stage reflectron TOF mass spectrometer may give further options (Berkenkamp et al., 1998). Although most of the bench-top ESI detectors have a mass range of 2-4 kDa, fragments of higher mass can be analysed using multiple charge states (usually 10-100 kDa). ESI can easily be coupled to high performance liquid chromatography (HPLC) or capillary electrophoresis (CE), which provides a separation or sample clean up of complex matrices. Further structural information on the parent ion generated by MS can be obtained by using tandem mass spectrometry (MS-MS). The mechanism of this technique, together with its applications, is described in detail in literature (Smyth, 1999). The mass of DNA is directly analysed and therefore, mass spectrometry offers increased sensitivity over conventional molecular biology detection methods. This technique offers great potential for automation and high throughput, yet is very costly and sensitive to the presence of contaminants.

DNA sequencing by mass spectrometry is far less advanced than protein sequencing. One reason is certainly that alternative well-established methods for DNA sequencing, such as gel-based or capillary electrophoresis already exist. In addition, DNA sequencing by MS is restricted to relatively small fragments. However, DNA fragments up to 160 bp have been recently sequenced by MALDI-TOF (Taranenko et al., 1998). Genotyping of short DNA fragments has been demonstrated also by ESI-MS (Laken et al., 1998). A complete genotype assay has been performed in less than 50 minutes by using the combination of a micro fabricated PCR instrument and delayed extraction (DE) MALDI-TOF-mass spectrometry (Ross et al., 1998).

It has been demonstrated that DE-MALDI-TOF-MS is an accurate analytical tool capable of characterizing a wide range of polymorphisms in a direct manner. A specific 89 bp nucleotide portion and a 114 nucleotide region of ribosomal rRNA genes have been amplified from the genomes of three members of the *Bacillus cereus* group and *Bacillus subtilis* and have been subsequently characterized by ESI Fourier transform ion cyclotron resonance (FTICR) mass spectrometry using a novel scheme for purification of PCR products prior to the detection (Muddiman et al., 1997). This technique has shown to be promising for the accurate and rapid evaluation of genetic variations. The high MS sensitivity may even decrease the number of amplification steps and further reduce the time for the analysis. Further investigations by the same authors have demonstrated that the use of modified bases in the PCR reaction may allow for modest mass measurement precision. The method has the potential to characterize PCR products of increasing size and could be used for rapid determination of base composition in the PCR products.

3.2 PROTEIN ANALYSIS METHODS

The specific detection of a novel protein synthesised by a gene introduced during transformation constitutes an alternative approach for the identification of genetically modified plants. Steinkeliner and Korschhineck (1998) provide an overview on recombinant proteins expressed in plants. It should be noted, however, that genetic modification is not always specifically directed at the production of a new protein and does not always result in protein expression levels sufficient for detection purposes. In addition, certain proteins may be expressed only in specific parts of the plant (tissue-specific promoters are already being used for specific purposes) or expressed at different levels in distinct parts or during different phases of the physiological development. The expression levels of transgene products in plants were reported to be in the range of 0 to 2% of the total soluble protein, even when strong constitutive promoters were used to drive expression (Longstaff et al., 1995). In most cases, however, the expression levels reported (e.g. for approved GM crops) are lower than the upper limit of 2% (Hemmer, 1997). Protein detection methods are based mainly on immunoassays. Since they

require proteins with an intact tertiary or quaternary structure, these methods are limited to fresh and unprocessed foods.

3.2.1 IMMUNOASSAY

3.2.1.1 Principle

Immunoassays are analytical measurement systems that use antibodies as test reagents. Antibodies are proteins produced in the serum of animals in response to foreign substances (antigens) and specifically bind the substance that elicited their production. In the case of detection of GMOs, the antigen can be the newly synthesised protein. Prerequisite for the development of immunological detection methods is that highly specific antibodies directed against the protein to be detected are available. In addition, the sample or the protein of interest should not be significantly degraded or denatured.

Immunological techniques have become indispensable tools in the physiological, biochemical and molecular disciplines of plant science. Their principal attraction resides in the high specificity of the immunological reaction, which allows accurate recognition of an antigenic substance even in the presence of interfering compounds. The methodology is now routinely used for the rapid purification, visualization and quantification of proteins, polysaccharides and even small molecules (haptens), in which case specific production of antibodies is induced by conjugation with an immunogenic carrier protein. Dumbroff et al. (1993) provide an extensive overview of immunological methods for assessing protein expression in plants. Brett et al. (1999) describe the design and development of immunoassays for the additional detection of proteins in novel food. Moreover, in a recent review of GMO detection methods applied to tobacco, the Task Force Genetically Modified Tobacco – Detection Methods of CORESTA (Cooperation Centre for Scientific Research relative to Tobacco, Paris, France) (Bindler et al., 1999) has highlighted the advantages and drawbacks of immunoassay methods used for GMO detection.

The most common type of immunoassay is the Enzyme-Linked ImmunoSorbent Assay (ELISA), which utilises an enzyme-labelled immunoreactant (antigen or antibody) and an immunosorbent (antigen or antibody bound to a solid support). A variety of methods (e.g. competitive binding between the labelled reactant and unlabelled unknown [e.g. a sandwich technique in which the unknown (an antigen) binds both the immunosorbent and labelled antibody] may be used to measure the unknown concentration of the antigen (Clark and Adams, 1977) (Figure 4).

The ELISA technique has been widely applied for evaluating, at the experimental stage, the expression level of the protein(s) synthesised by the newly introduced gene. Information regarding production and use of specific antibodies can be therefore found in many articles describing the developments of transgenic plants.

Several immunoassay-based methods, mainly ELISA, have so far been developed that are specific for gene products widely expressed in transgenic plants such as the neomycin phosphotransferase II (nptII) gene product, the enzyme 5-pyruvylshikimate-3-phosphate synthase (EPSPS), the *Bacillus thuringiensis* (Bt) insecticide Cry1Ab, and herbicide-tolerance phosphinotricin acetyltransferase (PAT) protein. The nptII protein has been expressed and purified from genetically modified cottonseed, potato tubers and tomato (Fuchs et al., 1993, Rogan et al., 1992, Wood et al., 1995). A modified ELISA method also based on the detection of nptII has been successfully applied to a number of independently transformed lines in nine plant species (McKenzie et al., 2000). The enzyme 5-pyruvylshikimate-3-phosphate synthase (EPSPS) has been demonstrated to be expressed in Roundup-Ready® soybeans (Padgette et al., 1995) for which an appropriate immunoassay method has been developed (Rogan et al., 1999). A direct sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) (see figure 4b) for the specific detection of the protein CP4-EPSPS in Roundup Ready®

soybean has also been tested and validated by Lipp and Anklam (2000). Preliminary results indicate that the method (performed by using a commercialised ELISA kit) is able to detect the presence of GMOs in raw soybean material at concentrations ranging between 0.3% and 5%.

However, differences may be observed in the expression level of the protein between crop varieties. Furthermore, the quantification of Cry proteins is made more challenging by the fact that EPSPS is naturally expressed in all plant species and that many different Cry proteins are used for genetic modification. However, new kits have been developed that are able to detect specific proteins in food crops, such as Bt Cry1Ac, Cry1C, Cry3A, Cry2A, Cry9C, CP4 EPSPS, and PAT.

ELISA methods offer a high degree of automation and sample throughput. Field variants of this technique, such as lateral flow strips or dipstick kits, have been recently developed and offer a semi-quantitative test of considerable practical value for field testing with simple laboratory set-up (Stave, 1999).

3.2.1.2 Pitfalls

One of the major drawbacks of immunochemical assays is that their accuracy and precision can be adversely affected in complex matrixes, such as processed vegetal and food products. Indeed, many substances present in food matrixes such as surfactants (saponins), phenolic compounds, fatty acids, endogenous phosphatases, or enzymes may inhibit the specific antigen-antibody interaction. Moreover, detection capability may be hampered when the transgenic protein is expressed at a very low level, or degraded and denatured by thermal treatment. The newly expressed protein may not be evenly present in all tissue of the plant. For instance, in maize the highest expression values for some proteins were mostly observed in leaves and not in grain. Finally the commercially available antibody may display poor binding affinity for the protein of interest.

3.3 ALTERNATIVE TECHNIQUES FOR GMO ANALYSIS

3.3.1 CHROMATOGRAPHY

Where the composition of GMO ingredients, e.g. fatty acids or triglycerides is altered, conventional chemical methods based on chromatography can be applied for detection of differences in the chemical profile. This has been demonstrated with oils deriving from GM canola for which high performance liquid chromatography (HPLC) coupled with atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) has been applied to investigate the triglyceride patterns (Byrdwelland Neff, 1996). The spectral identification was based on the diacylglycerols fragments and on the protonated triglyceride molecular ions. Quantification was performed with a flame ionisation detector (FID). In comparing the triglyceride patterns, it could be observed that the oils of the GM canola varieties had an increased content of triacylglycerols, showing more oxidative stability for high stearic acid canola oil as well for high lauric acid canola oil. This result is consistent with previous oxidative stability studies on new varieties of soybean and high oleic acid canola oils obtained by using HPLC-FID (Neff et al., 1992; Neff et al., 1994). In addition, the fatty acid compositions have been measured by using gas chromatography (GC) coupled with FID to support the HPLC results.

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

3.3.2 NEAR INFRARED SPECTROSCOPY

Certain genetic modifications may alter the fibre structure in plants, whereas no significant differences could be observed in the content of protein and oil (e.g. Round-up Ready (RR) soy beans). These could be detected by near infrared spectroscopy (NIR) as reported by Hurburgh et al. (2000). Sample sets of RR- and non-RR-soy beans have been used to develop discriminate analysis calibrations for various models of near-infrared spectrometers. The results obtained by the three NIR instruments varied slightly, but were promising in all cases. However, the capability of NIR to resolve small quantities of GMO varieties in non-GMO products is assumed to be low as is true for the chromatographic methods.

3.3.3 MICROFABRICATED DEVICES AND MICROCHIPS FOR DNA ANALYSIS

One of the challenges that the GMO analyst will face in the near future is the rapid pace of development of GM plants that feature new and multiple genes/genetic control elements. For example, Hemmer (1997) has already reported that some approved GM crops contained neither the 35S promoter nor the nos terminator. While the establishment of "gene registers" (see Section 2) and the use of advanced bioinformatic systems can help in obtaining prior knowledge of the possible types of genetic modifications, new technologies and instruments will be needed for the high throughput and low cost detection of an increasing variety of genes. New technologies resulting from the merger of chip-based microsystems such as microarrays and microfluidic systems (Sanders and Manz, 2000) appear to be a promising area for GMO analysis applications.

Microarrays have been used for expression analysis, polymorphism detection, DNA sequencing and genotyping (Lemieux et al., 1998). Microfluidic systems have applications ranging from reactions to separations and analysis and may finally lead to the development of micro Total Analysis Systems (mTAS) that perform the complete analysis including sampling and sample pre-treatment.

The microchip technology aims at automating the complex work procedures of an analytical laboratory by transfering them onto a small piece of glass or plastic, the so-called chip.

For example, in a microarray-based system, microscopic arrays of DNA molecules are immobilised upon a solid support (Souteyrand, 1999). Based on the principle of DNA hybridisation followed by monitoring, generally with fluorescence measurements, simultaneous analysis of several thousand nucleic acids within the very small area of a chip is possible. Therefore, the microarray system saves time and costs while maintaining high precision and reproducibility.

In a microfluidic system it is possible to simulate pumps, valves, reaction tubes and even analytical instruments by a clever transportation of liquids through miniature channels (5-20 mm) arranged on a single chip. Among the advantages are increased performance (e.g. more rapid cooling and heating times, more rapid diffusion across the channels, improved speed of separation), valveless transport (electro-osmotic or electrochemically driven flow), reduced consumption of reagents, portability, the possibility of parallelisation of procedures and high sample throughput.

Although several authors have reported on PCR-microsystems of different complexity (Khandurina et al., 2000; Ibrahim et al., 1998; Waters et al., 1998), only few examples of microchip applications to GMO analysis have been described so far (Moehrle et al, 2000; Kok et al., 2000) However, this new technology may offer new perspectives in the field of GMO detection.

In a microchamber PCR system various wells are connected to one channel which is to be flooded with DNA. Each well initially contains polymerase, deoxyribonucleotides and a different set of primers, so that different areas of the DNA can be amplified (Sanders and Manz, 2000). The whole system is thermally cycled with improved thermal diffusion compared to macrostructures and thus enabling faster cycling.

In a chip thermocycler a continuous flow of DNA material proceeds in miniaturized channels through well-defined zones with different temperatures required for melting, annealing and extension (Sanders and Manz, 2000). The chips have been designed for as many as 20 cycles with a total cycling time of about 10 min.

PCR and Capillary Electrophoresis (CE) have been integrated into one microfabricated analysis device, thereby allowing reaction, separation and analysis at once (Woolley et al., 1996; Waters et al., 1998; Ibrahim et al., 1998; Khandurina et al., 2000).

More recently, miniaturization of PCR technology that includes a DNA extraction microdevice has been achieved within biowarfare defence research programs. This has allowed the development of hand-held combined instruments capable of performing rapid DNA analysis on-site. Belgrader et al. (1998,1999) reported the development of a battery-powered miniature analytical thermal cycler instrument (MATCI), i.e. a silicon chip-based spectrophotometric thermal cycler capable of performing real time quantitative PCR. The entire instrument, including a laptop computer, is housed in a briefcase and can be carried by hand. It gives a preliminary, rapid (20 - 30 min) assessment of the likelihood that a particular bio-agent is present

A product called Smart Cycler® XC (Xtreme Conditions) System (Cepheid, Sunnyvale, CA) is a very rapid battery-operated thermal cycler with real time optical detection, featuring all reagents for sample preparation and processing in a disposable cartridge with microfluidic components. Once the cartridge is loaded, the sample (microlitre to millilitre amounts) is chemically processed and the DNA is extracted, concentrated, amplified and analysed. Thermocycling, real-time fluorescent detection (can use up to four different fluorescent dyes) in a Cepheid I-CORE® module, and reporting of results take place in the hand-held base unit within 30 minutes from sample loading. The microfluidics technology is capable of accepting large volume biological fluid samples, automatically extracting, purifying, and concentrating DNA or RNA, mixing the target DNA/RNA with the appropriate amplification/detection reagents, and presenting the final mixture to the amplification process.

Besides applications in rapid pathogen detection in foods or for point-of-care human diagnostics, the use of microPCR portable systems can be envisioned in field detection of crop disease and point-of-use GMO screening, e.g. in the early steps of the supply chain, without the need for an expensive laboratory setting. It can also be envisioned that the integration of advanced diagnostic micro instrumentation and Internet technology may be employed in the future to develop wireless and mobile GMO detection instruments that can readily identify plant genetic modifications on site. Research groups at the University of Tampere and University of Turku in Finland have recently developed a new bioinformatic service (called BioWAP, see http://www.uta.fi/imt/bioinfo/BioWAP/) for portable devices such as mobile phones, which is based on WAP (Wireless Application Protocol), a global standard for mobile Internet connections and available from all the major mobile phone manufacturers. BioWAP provides mobile phone access and searching facilities to more than 20 major nucleotide and protein sequence databases (e.g., EMBL, GenBank, SwissProt).

Most of the reviewed microfabricated devices take advantage of the capability of PCR to amplify low copy number targets from a background of non-specific nucleic acids. However, the development of PCR protocols for GMO analysis is often affected by misspriming due to polymerase errors that results in the extension of non-specifically annealed sequences and the production of undesirable PCR artefacts. Moreover, in GMO analysis of complex food matrices, inhibitors of Taq polymerase often co-purify with the DNA template and lead to false negatives. Although PCR and its many variations and formats still remain the most sensitive and widely used

DNA diagnostic technology, non-PCR detection methods and microfabricated devices have recently been described which allow genetic analysis in conditions of low abundance of target fragments.

One microtechnology that can be applied for both DNA and protein analysis is surface plasmon resonance (SPR) (Rich and Myszka, 2000). In particular, SPR has emerged as a well-suited method to study the real-time kinetics of biomolecular interactions between macromolecules in a label-free fashion, e.g. antigen–antibody interactions, protein–DNA interactions and receptor–ligand interactions.

SPR stems from one of the basic principles of optics, the one of total internal reflectance, and occurs when a thin conducting film is placed at the interface between materials with differing refractive indices. If a ligand can be conjugated to the surface of the biosensor chip, then the attachment of a free target analyte present in solution can be measured as a function of mass increase. The change in angle of reflected light (proportional to the difference in mass on the chip surface) before and after incubation is recorded in a "sensorgram" and measured in relative units (R.U.). Accordingly, changes in response levels in spiked samples can be correlated with known analyte concentrations.

Kai et al. (1999) have employed a flow-injection type of SPR for the sequence-specific detection of the gene encoding the verotoxin subunit A of E. coli 057:H7. This gene was used as a template for an asymmetric PCR reaction in order to produce a 3'-protruding DNA amplicon that hybridises to a probe immobilised on the surface of a chip.

Minunni et al. (2000a) have proposed the use of biosensor technologies, including SPR for screening purposes in GMO analysis. They have highlighted several advantages of this technology including fast time responses, ease of use and low costs. These investigators obtained preliminary good results with an electrochemical biosensor featuring 35S promoter and nos terminator oligonucleotide probes immobilised as capturing agent on a screen printed electrode. The probes recognized the complementary DNA sequences when exposed to the target analyte in solution, with the system being much more sensitive to the 35S than the nos target. The reference analytes were synthetic oligonucleotides and PCR amplified DNA samples from RR soybean Certified Reference Material (Fluka). Similar results were obtained when the same probes were immobilised on the surface of a SPR sensor (BIAcoreTM) (Minunni et al., 2000b).

Taton et al. (2000) at Northwestern University (Evanston, IL, USA) developed another non-PCR detection micro technology called "scanometric DNA array detection". This technique uses DNA arrays and a signal amplification method based on gold nanoparticle probes covered with 200 oligonucleotide strands that are complementary to a target DNA sequence immobilized on a chip. When the nanoparticle probes bind to target DNA they polymerise and form structures containing thousands of particles. The signal is amplified using a modified photographic developing solution that covers each gold nanoparticle with silver while the network of particles grows in size, thus increasing the signal by a factor of 100,000. The imaging of the detection reaction is performed with a simple flatbed scanner. The authors reported higher and sharper DNA temperature melting profiles than those found in conventional detection methods based on fluorescence technology, with the ability of detecting as few as 60 DNA molecules without the need for a PCR amplification step.

3.3.4 NANOSCALE GMO ANALYSIS

The enforcement of GMO regulations, including the recently introduced labelling and threshold provisions, require the accurate determination of very low amounts of transgenic DNA in a sample. EU regulations allow for the inadvertent co-mingling of GMOs to the extent of 1 % at the single ingredient level. Moreover, some customers and consumers are unwilling to accept any GM or GMO-derived product with disregard to the level of transgenic DNA or protein possibly present in the food sample.

Single cells isolated by flow cytometry or micromanipulation can provide sufficient amounts of DNA to allow PCR detection (Schwers et al., 2000), although amplification of single-copy genes may be particularly challenging. Single cell PCR enables to study, for example the variations in gene expression between individual cells in a population, in gene expression between different cell types at different developmental stages, or can be used for early diagnostics purposes in human diseases.

Single molecule detection (SMD) (Ishii and Yanagida, 2000), for monitoring the chemical and physical properties of biochemical reactions of individual molecules in real-time, represents the most advanced frontier in analytical technologies, and is now capable of visualizing the elementary molecular dynamics processes that occur in the timescale of femtoseconds (1 fs=10^{-15} s) to picoseconds (1 ps=10^{-12} s) (Chergui, 2000). Among the techniques used in SMD are laser-induced fluorescence spectroscopy (either on surfaces or on freely moving molecules in liquids), and magnetic resonance. SMD was recently applied to ultra fast DNA sequencing, capable of identifying the four nucleotides in a DNA-sequence according to their fluorescence lifetime. In this method, a single strand of DNA is replicated in the presence of fluorescent-labelled deoxynucleotides (dNTP). The DNA polymerase makes a faithful copy of the original DNA but with fluorescent labelled nucleotides. A single molecule of this DNA strand is then attached to a small bead by biotin-streptavidin contact. The bead is caught and held in a laminar flow inside the microstructure with the aid of an optical tweezer. An exonuclease enzyme is finally added to cleave the DNA and each dye-marked nucleotide is released and transported with the flow to an optical detection zone. As the molecule passes through the detection zone, a burst of fluorescence is generated and detected by avalanche photodiodes. This method of DNA sequencing presents several potential advantages over conventional sequencing technologies, for example the possibility of sequencing much longer DNA strands at higher speed, since the exonuclease can cleave many nucleotides per second.

A promising future perspective for SMD appears to be the detection and direct quantification of trace amounts of DNA by counting single molecules in sub-microlitre volumes of liquid, possibly leading to the development of “nanoscale GMO analysis” techniques.

4 A NEED FOR HARMONIZED METHODOLOGY

The development and application of reliable detection and quantitative analytical methods is essentials for the implementation of labelling rules. Appropriate sampling strategies and methods for sample preparation, as well as methods development and application to detect GMOs and their derivatives must then be followed by the establishment of validation criteria and performance assessment. Since reference samples are an indispensable part of any analytical protocol, criteria for manufacturing standard reference materials must also be considered. This part of the review discusses the aspects that need to be considered while establishing criteria for sampling, sample preparation, and result interpretation and covers the procedures for validation of PCR methods and immunoassay analyses.

4.1 SAMPLING

The sampling procedure determines the "representativity" of a result, whereas quality and quantity of the analytes may vary depending on the sample preparation. Sampling and sample preparation are thus crucial steps in the process of GMO detection (Terry and Parkes, 2000). The limit of detection of the analytical method as a whole is determined, not by the most sensitive part of the procedure, but by the least. In most cases this is the sample size.

4.1.1 HOMOGENEITY OF THE SAMPLE AND SAMPLE SIZE

A sample has to be representative of the batch/lot of the product from which it was taken, and the sample plan and sample size have to meet statistical requirements with respect to homogeneity and threshold limit up to which the result should be reliable. Therefore on the one hand, the sampling plan must ensure that the field sample is taken in a statistically representative manner with respect to the larger lot of material and, depending on the sub-sample size desired, the particle size distribution should be such that a sufficient degree of homogeneity is achieved. On the other hand, the sample size must be sufficiently large to allow reliable detection at the desired sensitivity. Sample representativity must be maintained during subsequent reduction of the field sample to laboratory and test samples. Finally, sampling, sample size and homogenisation must follow a protocol that can distinguish between surface contaminated seeds and contaminating seeds. This is particularly relevant for the detection of non-authorised GMOs.

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

Raw materials are often not systematically mixed during harvest, storage, etc., resulting in strata that can seriously invalidate assumptions associated with simple random sampling,

Ingredients are processed and thus already present at restricted degree of variance. Nevertheless, different batches may also present different characteristics,

Processed foods should contain GM material only as a source of one or several of various ingredients, so a potentially strongly stratified variance distribution can be expected in many cases. However, within each ingredient of the processed food, variance should be low.

The degree of homogeneity of a given sample and the actual threshold limit which is set for acceptance of the presence of GM material will define (i) the number of samples to be taken and (ii) the appropriate sample size. The lower the degree of homogeneity, the more critical will be the choice of the appropriate sampling plan. Moreover, when only low levels of GM material are acceptable, the required sample size will increase accordingly, in order to be representative. For the sampling of food commodities, there is already experience with well-tested sampling plans for

analogous detection problems (Gilbert, 1999; Berger and von Holst, 2001; Whitaker et al., 1995; Grain Inspection Handbook, 1995).

As already discussed before, if the maximum desired concentration of GM-seed in a lot is 1%, a single sample size of 299 kernels – producing a negative result - is sufficient to assure that this threshold is not exceeded at a 95% confidence level. If a 0.1 % concentration is the desired maximum, the sample size should be about ten times higher (~3,000). These considerations are based on the assumption of a standard binomial distribution, a reasonable premise if appropriate systematic sampling is conducted assuring an equal probability of selection for the lot.

Several other authors have suggested similar sample-size-estimations according to statistical considerations that are based on the assumption of a homogeneous distribution: A lot can be accepted with 95% confidence when a sample size of approximately 300 grains is subjected to analysis and no GMO is detected. The acceptance implies that the lot may contain a maximum of 1% GMO. (Ryman, 1999; von Holst, 2000)

Two further references describe a different sample size:

The Working group "Genetically Modified Foodstuffs" within the Technical Committee CEN/TC 275 of the European Committee for Standardization (CEN, Brussels, Belgium) assumed that a sample size of 10,000 particles is representative with 95% confidence if a GMO content of 1% is expected (Heissenberger, 2000).

Hübner (2000) calculated that a sample size of at least 3,500 particles should be examined for an expected GMO-content of 1% in order to establish a result with 95% confidence and a sampling error lower than 20% (corresponding to a coefficient of variation = 20%).

The different sample sizes here reflect different assumptions and degrees of sophistication in the sampling strategy that can be feasibly applied. In addition, the requirement for quantification (or not) can seriously alter the sample size required; semi-quantitative detection methods demand considerably fewer grains to be sampled.

4.1.2 THRESHOLD LIMIT AND SAMPLING ERRORS

Scant attention is paid at present further down the analytical chain, in particular to the sampling of the laboratory sample (the DNA extracted from the bulk material) in the production of the analytical sample. A number of discussions and publications appear to overlook this fundamental scientific phenomenon, and mistakenly assume near-perfect, homogeneous distribution of GMO DNA copies in repeat sampling from laboratory samples. In reality it exists a lower limit to the limit of detection for any analytical system – PCR or protein-based - created by the unitised behaviour of DNA copies, and the statistical nature of random sampling.

The DNA content of the unreplicated haploid complement, known as the 1C value, can be used as an intrinsic factor that relates the number of genome copies to amount. As previously explained, up to 36 697 copies would be present in a typical 100ng analytical sample of *Zea mays*, given the 1C-value of 2.725pg (Bennet et al., 2000). This implies that for such a sample, the presence of a single GMO copy would represent a w/w percentage of 0.0027% (Cochran, 1977). For a 100ng sample, therefore, this becomes the lowest concentration theoretically possible; absence of detection in a lower concentration laboratory sample would thus be a statistical artefact, and not a valid result.

If the laboratory sample is large (say, 50μg), the selection of the analytical sample (100ng) can be made according to simple random sampling procedures, with replacement (Forthofer and Sul Lee, 1995). However, even in conditions of perfect homogeneous preparation of the laboratory sample, a

"sampling error" will occur. In particular, when the concentrations concerned are low, the sampling error becomes (proportionally) larger. When calculating the range of the expected number of copies in the analytical sample for estimates of GMO concentrations (expressed as w/w) of 0.1%, analytical samples of 100ng would produce no better than +/- ~30% of the mean value, 95% of the time. Furthermore, given that this is purely sampling error, degradation in this result can be expected due to the imperfect behaviour of a real analytical system.

It is possible to calculate, using the well-understood cumulative distribution function for the binomial distribution (Biotecon Diagnostic GmbH., 2000), the probable range of number of GM copies that would be "sampled" in a single-step procedure, i.e. from a (large) laboratory sample of "known" low concentration (0.1% GMO) into a series of 100ng analytical samples (see Table 6). While on average, the analytical samples should contain 36.7 GMO copies; in fact the number of GMO copies would range from 25 to 48, with a 94.3% confidence level. Thus the actual concentration that would be observed in a single sample, with an approximate 95% confidence level, would range from 0.068% to 0.131%; the probability of sampling exactly 36 GMO copies in a single analytical sample is only 0.066 %. With lower concentrations, the situation is more critical. For a laboratory sample of 0.01% concentration, the 100ng analytical sample would vary between 0.0027% and 0.0191% nearly 95% of the time (Kay et al., 2001). These calculations obviously refer to a "best possible" result, since they assume a single sampling step and a perfect analytical system.

When undertaking a dilution series, it is important to note that the assumption of sampling with replacement may no longer be valid, since the number of copies available becomes strictly finite. Indeed, the number of copies used to prepare subsequent dilutions heavily influences the sampling error associated with the series. Consequently, the preparation of any dilution series must be undertaken in such a way so as to minimize this bias; ideally, dilutions should be made from the primary laboratory sample. The classical solution to the issue of sampling error is to undertake repetitions, and/or use appropriately sized (i.e., larger) analytical samples. We recommend that in the construction of a dilution series - for example for determination of Limit of Detection of a method, or for the generation of standard curves - that the nominal number of GMO copies in the weakest dilution of analytical sample should be set to around 20, thus providing good statistical probability that all repetitions contain relevant DNA (Table 6). However, we are aware of important studies that seem to draw conclusions without such safeguards, despite explicitly working with copy numbers. In conclusion, there is insufficient acknowledgement in the domain that repeated analytical samples drawn from a "homogenized" laboratory sample would not have identical proportions of GMO/non GMO copies.

4.2 PRINCIPLES OF METHOD VALIDATION

Analytical methods used by enforcement laboratories, especially where legal proof may become necessary, should be subject to validation procedures, in order to show that the method produces reliable and repeatable results. The objective of the method validation is to demonstrate that the defined system (which may include various steps in the analytical procedure, and may be valid for a restricted matrix) produce acceptably accurate, precise, and reproducible results for a given analyte. The process of validation allows the independent use of methods and produce results that are comparable between different laboratories.

Depending upon the intended purpose of the analysis, i.e. qualitative screening or quantification, different validation parameters have to be evaluated. Moreover, these studies must be carried out according to harmonised international protocols (e.g. IUPAC, AOAC, ISO), and cover as wide a range of laboratories as is feasible, usually considered to be no fewer than eight.

However, since large-scale collaborative studies require a considerable level of effort and resources, they should be conducted only on those methods that have undergone appropriate pre-testing (Figure 5). The objective of this preliminary work is to define the performance characteristics of the method, and to set target values for the parameters to be evaluated in the validation trials. Alternatively, a method performance study could be used to establish if the claimed performance of a method (or, more commonly, test kit) is as claimed by a manufacturer. Typically, these data are collected either in-house, or through small scale multi-laboratory studies, which provide information on the expected precision (within laboratory standard deviation), possible systematic error (bias), recovery values (on the basis of spiked material), applicability, interference with other compounds during analysis and best calibration approaches (Horwitz, (1995). The output from this stage is the detailed method description (standard operation procedure) to be distributed to the participants in the actual validation trial study, along with a series of expected parameter values with which the full validation study results can be compared. Such procedures to validate method performance and results of analysis of contaminants and residues have been described in detail elsewhere (International Conference on Harmonisation, 1996; Ambrus, 1999). Validation parameters for qualitative and quantitative methods are listed in Table 7.

The choice of the parameters to be explored during the validation process is determined by the intended use, as well as the nature of the method in question (Schofield, 2000). Typically, GMO detection will require most parameters to be carefully defined, due to the unknown concentrations of the analyte in the test samples, combined with the increasing demand to detect at lower levels and for comparison of results with labelling thresholds.

Specificity, sensitivity, detection limit, matrix effects/inhibition, precision (repeatability, intermediate precision, reproducibility) and robustness have to be established for identification/screening purposes (qualitative detection). In addition, accuracy, quantification limit, linearity, working range, coefficient of variation and measurement of uncertainty have to be evaluated for a quantitative detection system (EMEA-guidelines CPMP/ICH/281/95 and 381/95; Anklam, 1999; Hübner et al., 1999b; Köppel-Overkamp, 2000).

The requested parameters for a qualitative method, i.e. test for presence or absence of the analyte, can be summarized as follows:

Specificity: is defined as the probability to obtain a negative result given that the analyte is not present (Forthofer and Sul Lee, 1995). It can be established by determining the percentage of correct classification as GMO negative of a non-analyte-containing sample. According to the guidelines published by the Paul Ehrlich Institute (PEI) (1998), at least 100 different negative controls should be tested in order to establish specificity of the primers. The identity of the amplicon should then be further controlled by additional characteristics (exact size, sequence, restriction profile, probe-hybridisation).

Sensitivity: is defined as the probability to obtain a positive result given that the analyte is present (Forthofer and Sul Lee, 1995). It is established by determining the percentage of correct classification as GMO positive of an analyte-containing sample. According to the PEI guidelines (1998) the exclusion of false negatives, e.g. an inhibition due to matrix effects, can be controlled by the co-amplification of an internal control. Two additional controls should be used routinely: a negative control as a test for contamination and a positive control close to the detection limit as a test for sensitivity.

Limit of detection (LOD): is determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can reliably be detected. According to the EMEA-guideline CPMP/ICH/281/95 the LOD can be described as

LOD = (3.3 sigma): S

Where “sigma“ is the standard deviation of response and „S“ is the slope of the calibration curve. According to the PEI guidelines the detection limit can be defined as the concentration at which 95% of the experiments give a signal (i.e. 95% sensitivity) and should be experimentally determined by at least three series of dilution in DNA background where each dilution should be analysed in eight replicates.

Precision (repeatability, intermediate precision and reproducibility): the intra-laboratory variation should be determined by repeating the experiments over a short time (repeatability) and over a prolonged time period, i.e. on different days with different staff and different equipment (intermediate precision). The inter-laboratory variation needs to be determined in a ring trial (reproducibility).

Robustness (stability of the method): the reliability of an analysis should be demonstrated with respect to deliberate variations in method parameters (detailed work instructions, storage conditions and stability of analytical solutions).

Overall accuracy (OA) (sometimes called “reliability“): can be defined as the probability to obtain a correct result. It is established by determining the percentage of correct results of a given number of tests. If sensitivity and specificity have already been determined the OA does not provide any further information about the method. However, some of the validation studies discussed below did publish data only allowing a rather rough assessment of their performance by the OA value.

According to the EMEA guideline CPMP/ICH/281/95 (1996) a quantitative analytical method should additionally evaluate the following parameters:

Accuracy: should be reported (i) as the percent recovery by the assay of known added amount of analyte or (ii) as the difference between the mean and the accepted (true) value of the reference material together with the confidence intervals.

Limit of Quantification (LOQ): is determined by analysis of known samples and establishes the minimum level at which the analyte can be quantified. The LOQ is described as

LOQ = (10 sigma): S.

Sigma“ and “S“ are defined as previously described (see above, limit of detection).

Linearity and working range: the proportionality of the signal to the amount of reference material should be demonstrated by the calculation of a regression line with the adequate statistical method. The linear range thereby obtained may define the working range, i.e. where the measurement is done in routine.

Precision: has to be demonstrated in terms of repeatability, intermediate precision and reproducibility (see above). The recommended data include standard deviation, relative standard deviation (coefficient of variation) and confidence interval.

4.3 RING TRIALS AND COLLABORATIVE STUDIES ON DNA METHODS

4.3.1 PCR METHODS

Irrespectively of a variety of potentially available methods for DNA analysis, so far only PCR in its different formats has found broad application in GMO detection/analysis and has been generally

accepted for regulatory compliance purposes. Indeed, an increasing number of food control laboratories are adopting PCR as the technology of choice for GMO (qualitative) detection and (quantitative) determination. The methods used can be classified into three groups: qualitative PCR detection methods for screening, qualitative PCR methods for identification of GMOs, and quantitative PCR methods for determination of GMOs content. By contrast, current ELISA methods cover aspects of both screening and quantification and will be discussed in more details in the part dedicated to protein analysis (section 4.4).

The adoption of official or validated methods as an analytical tool for GMO detection in raw plant materials or plant-derived foods is in its initial stage. An overview of the trials performed so far is given in Tables 8 to 11 (Anklam et al., 2002).

For the PCR methods, the comparison of performance of the evaluated methods is to some extent possible on the basis of overall accuracy. Other validation parameters, such as specificity and sensitivity, which would provide more information on the accuracy of the analytical procedures, were included in only few of the ring trials reports. Moreover, for the performance assessment of quantitative detection methods, various statistical approaches were utilised. Differences in the experimental design (for example, number of laboratories, sample concentrations, etc.) and lack of direct access to the data necessitate caution in evaluating performances between different ring trials.

4.3.1.1 Qualitative PCR detection methods for screening

PCR detection methods for qualitative screening (Table 8) were assessed by the use of the Swiss method on soybean raw material and Soya lecithin (Brodmann et al., 1997), and were the subject of two other European ring trials coordinated by the Joint Research Centre (JRC) (Lipp et al., 1999; Lipp et al., 2001).

The Swiss study (Brodmann et al., 1997) evaluated 6 laboratories, each provided with eight samples of soybean raw material and lecithin, ranging from 0.1% to 1% concentrations, tested in duplicate. The method is based upon the detection of the 35S promoters and *nos* terminator. The report (Brodmann et al., 1997) shows favourable results, with overall accuracy reported as greater that 90%.

The two screening methods ring-trialed by the JRC (Lipp et al., 1999; Lipp et al., 2001) were, like the official Swiss method, based on the detection of P-35S promoter and *nos* terminator by PCR. The participants of the trial were free to apply their method of choice for DNA extraction, were requested to optimise the PCR conditions for their own equipment, and were free to purchase their reagents locally; to some extent therefore, this trial has some elements of a proficiency study. The primer sequences were the only parameter on which modifications were not allowed, thus serving as a pivotal point in these studies. The PCR products were separated by electrophoresis and examined by comparison to length standards expecting a 195 bp amplicon for the P-35S promoter and a 180 bp amplicon for the nos terminator. The identity of the amplicons was confirmed by restriction enzyme analysis. The blind-coded samples tested ranged from 0.15 to 2%, with an average of 4 samples for each concentration and matrix being tested in each lab. The performance in both ring trials was again positive with an overall specificity (i.e., correctly classified as GMO negative) above 96% and an overall sensitivity (i.e., correctly classified as GMO positive) above 93%. Although several performance parameters are not reported directly, LOD can be determined as being between 0.1% and 0.5%.

Concerning the validation of screening detection methods on non-food crops, the Task Force Genetically Modified Tobacco – Detection Methods of CORESTA (Cooperation Centre for Scientific Research relative to Tobacco, Paris, France) (Bindler et al., 1999) recently initiated a

collaborative study to evaluate the performance of a dry tobacco leaf protocol adapted from the one validated by JRC (Gadani, 2000).

4.3.1.2 Qualitative PCR methods for specific identification of GMOs

The three ring trials for the official German methods (10) are based on the specific PCR-based GMO identification on raw materials: potato (LMBG 24.01-1, 1997), soybean (LMBG 23.01.22, 1998), and tomato (LMBG 25.03.01, 1999). The comparison that was made was based upon pure samples of the GMO in question (e.g. single soybeans). The reports include a description of procedures for DNA extraction (CTAB-method), PCR analysis and a confirmatory test. The PCR is performed with suitable primers specific for the GMO under study and the amplification capacity of the extracted DNA is determined by an internal control. The amplicons are examined by agarose gel electrophoresis and their identity is confirmed by Southern blot. The results of ring trials demonstrated overall accuracy (i.e., percentage of laboratories correctly identifying the sample) above 97%; however, the reports here do not interpret the data in detail and the main performance parameters are not presented.

The DMIF-GEN Final Project (1999) successfully ring trialed soy meal and maize flour samples from 0.1% to 2%, in two studies involving 16 and 18 laboratories, respectively. For soy, all 96 samples were correctly identified, and for 75 maize samples, only one false positive and one false negative result were communicated.

A study (DMIF-GEN Final Project, 1999) of the Federal Institute for Health Protection of Consumers and Veterinary Medicine, Germany (BgVV) on processed foods containing soy and maize (in concentrations of 0%, 2% and 100%) and involving 15 laboratories, produced an overall accuracy of 89%. However, a high false positive rate (11 of the 12 incorrectly assigned samples) indicates that a certain degree of contamination is the probable cause of this weaker result.

The Swiss trial (Brodmann et al., 1997) reported above also covered the specific detection of RoundupReady® Soybean, using a method based upon the GM07/GM08 primer pair; 96% overall accuracy was reported amongst the 88 results evaluated. Samples varied from 0.1 to 1% in concentration.

4.3.1.3 Quantitative PCR methods for determination of GMOs content

The ring trials on quantitative PCR methods (Table 10) include:

A collaborative study evaluated four quantitative competitive PCR methods on soybean flour and several commercial food samples containing Roundup Ready® soybean at unknown levels (Hübner et al., 1999b). The results were classified into four classes: negative, <0.5%, 0.5% to 2%, and >2% GMO. One method was abandoned due to lack of sufficient robustness; however, for the remaining three approaches, the results presented showed good correlations (contingency coefficients between 0.77 and 0.81) and consistent classification for the methods tested.

A semi-quantitative competitive PCR method for soy and maize meals and one PCR-ELISA method for soybean meals were evaluated in the DMIF-GEN Final Project (1999). The former method (based upon OD260 values) showed disappointing results for soybean; by contrast, the maize ring trial, involving 19 laboratories, gave a 97% overall accuracy for 2% and 0.1% samples while accuracy for the intermediate values of 0.5% and 1.7% dropped to 63% and 81% respectively. For the PCR-ELISA approach, samples of 1% and 2% were all correctly analysed by 11 of the (initially13) laboratories delivering valid results. Correct classification of the 0.1% samples, however, resulted to be more difficult.

Real-Time PCR methods for soybean meals were evaluated under coordination of the BgVV (EU Tender Report, 2000). Three equipment configurations were tested with similar protocols (Taqman™), in between 6 and 14 laboratories (depending upon the instrument used). Six samples were tested in duplicate, with the analysis showing overall Coefficient of Variation closed to 25% for the ABI 7700 instrument. For the ABI5700 and Roche LightCycler™ Coefficient of Variation higher than 40% were obtained.

A double competitive PCR method for raw material from soybean and maize was also evaluated under the coordination of JRC (Van den Eede et al., 2000). An overall accuracy of more than 70% was reported for 0%, 0.1%, 0.5%, 1%, 2% and 5% GM-soybean samples. The overall accuracy for 0%, 0.1%, 0.5%, and 1% GM-maize samples was also above 70%, however for levels above 1% GM-maize, the performance decreased significantly.

4.4 RING TRIALS AND COLLABORATIVE STUDIES ON PROTEIN METHODS

4.4.1 IMMUNOASSAY METHODS

Protein-based immunoassay methods (Table 11) often present a practicable alternative to PCR techniques, i.e. for on-the-spot GMO detection. Lipton et al. (2000) have discussed the validation and application of immunochemical methods for the analysis of transgenic products in vegetable and food derivatives. Future needs for detection of new or modified proteins in novel foods derived from GMOs are further described in Stave et al. (1999). As for PCR, there is a strong need for validated methods to make appropriate comparison of efficiencies.

Two ELISA kits have been validated so far by collaborative trial studies.

The performance of a diagnostic ELISA kit for detection and quantification of CP4 EPSPS from genetically modified Roundup-Ready® soybean has been assessed (Lipp and Anklam, 2000) in a European ring study performed under the coordination of the JRC. It has involved 38 laboratories from 13 Member States and Switzerland. In this validation study the ELISA assay gave an incorrect assignment of GM status in only 1% of the samples in which the GMO was present at a level of 2% or greater. The immunoassay demonstrated a good repeatability with RSD_r = 7%, a promising reproducibility with RSD_R = 10%, and a detection limit of approximately 0.35% GMO.

Another recent collaborative study was successfully performed (including 40 laboratories in 20 countries) to detect Mon 810 maize with a specific ELISA test based on the CRY1(AB) protein. The quantitative range reported was between 0.15 – 2.0 %, with a reproducibility (RSD_R) figure of better than 23 % (Stave et al., 2000).

4.5 DISCUSSION

Current legislation demands the labelling of food products when they contain more than 1% GMO at the ingredient level. In order to create confidence in the testing procedures and complement enforcement requirements, there is an urgent need for using methods that are validated and officially recognised at an international level. To date, several methods for different food matrices have been submitted to validation trials. Although encouraging, the resulting reports have presented rather limited statistical analysis, which suggests a more precautionary approach in their evaluation (see Tables 8-11). Indeed, the methods subjected to ring trials varied significantly in their reliability, robustness and reproducibility. Even though claims of very high sensitivity were sometimes made, they were often not supported by appropriate statistical analysis and detailed performance studies. GMO detection with analytical precision should involve the consideration of a number of critical parameters with respect to quality control. Any laboratory using such methods is expected to

carefully validate every single step of the analytical process in order to produce comparable results. An important point to consider however in the estimation of the sensitivity for the GMO analysis is that as previously discussed (see sections 3.1.3, 3.1.6 and 4.1.2) there is a lower limit to the limit of detection due the unitised behaviour of DNA and the statistical nature of random sampling. Indeed, the practical detection limit may be significantly lower; i.e. 0.1% GMO/non-GMO (w/w) than the one theoretically calculated for a PCR reaction; i.e 0.005% GMO/non-GMO (w/w) (Jankiewicz et al., 1999).

More validation studies, especially on the quantitative PCR methods, need to be performed to provide new extraction methods for food matrices and novel GMO constructs. Considerable work remain to be done, therefore, in response to the increasing demands of consumers and policy makers to give confidence that food products on the market meet the requirements of the rapidly evolving EU legislation.

4.6 INTERNATIONAL STANDARDIZATION AND VALIDATION OF GMO ANALYSIS METHODS

In the field of GMO analysis, validation has so far been based on collaborative studies, with some methods even ring-trailed at a global level. The European Committee for Standardization (CEN) and the French Standardization Association (AFNOR) are in the phase of producing guidelines for PCR methods for GMO detection and quantification (Heissenberger, 2000; AFNOR, 2000), which reinforce the international importance of this work. However, international standardization and validation of GMO analysis methods by harmonized and accepted protocols is still in its early phase. Standardization bodies such as the European Committee for Standardization (CEN, Brussels, Belgium) and the French Standardization Association AFNOR (Paris, France) have undertaken activities in this area and produced preliminary guidelines for sampling strategies and GMO detection methods, respectively (Heissenberger, 2000; AFNOR, 2000).

Moreover, in accordance with the Agreement on Technical Barriers to Trade in the multilateral trade negotiations that established the World Trade Organization in 1994, the International Organization for Standardization (ISO, Geneva, Switzerland) has taken the mission of assuring international harmonization of standards. These comprise standards for analytical methods (guidelines, norms and regulations) and accreditation services, and should guarantee that accredited laboratories with validated methods produce comparable results; thereby avoiding costly and time-consuming re-testing of shipments

4.7 PROFICIENCY TESTING

To demonstrate that the results of a validated method are correct, control laboratories should participate in proficiency testing, this experience being also a valuable step in the preparation for accreditation.

Participation in proficiency testing has become systematic for laboratories (especially official control laboratories) over the past few years. The various proficiency schemes are designed to assess qualitative results as well as quantitative analysis capabilities (see figure 5). Several proficiency-testing programmes have been established throughout the world with the aim to identify problem areas within a laboratory, to provide experience for the participating analysts and help with the implementation of quality control practices. In contrast to a validation study, a proficiency-testing programme does not specify which method will be used to determine the result. Nevertheless, the use of validated methods is an obligation for accredited laboratories. These tests are valuable tools for assessing the laboratory's analytical performance against a "best practice" benchmark. The

organisers of the proficiency programme prepare only the test materials, making sure that the latter are uniform. Ideally, materials should be spiked with the compound to be analysed in order to provide participating laboratories with a stable, uniform material of known composition and contamination, thus assuring that the results can be reliably compared to a reference value. Nevertheless, naturally contaminated material can also be used.

The results of such tests are returned by the participating laboratories, together with information about the method used, the calibration approach etc., to the proficiency test co-ordinator. The laboratories' performance is evaluated by comparing the results with the "true" value (spiked material) or the combined results of all other laboratories (relative approach). In the case of investigation of qualitative testing capabilities, the number of false positives and false negatives can serve as a basis for performance assessment. Several aspects of proficiency testing are discussed in detail in the literature (Mishalani, 1999). So far, only five proficiency tests on GMO detection have been publicly report within Europe, from the programmes co-ordinated by the Ministry of Agriculture, Food and Fisheries (MAFF) or the Food Standards Agency (FSA) of the United Kingdom (Howell, 2000; FAPAS, 2000).

4.8 REFERENCE SAMPLES AND CONTROLS

Reference samples are an indispensable part of any analytical protocol. These may be certified reference samples or may be internal standards, such as DNA preparations (Pawls et al., 1999a, 1999b). The Institutes for Reference Materials and Measurements (IRMM) provides certified reference samples for the validation of PCR screening methods intended to detect genetically modified food GMOs Roundup Ready Soya, Bt-176 maize and Bt-11maize (see Table 12). The CRMs are available with a certificate of validation for the PCR screening method. The validation procedure has been coordinated by the Environment Institute (EI) of the Joint Research Centre of the European Commission (Ispra, Italy) and has included 22 laboratories, where 352 unknown and randomly distributed samples of the four CRMs were analysed. Additionally, supplementary gravimetrically prepared samples are available. It should be noted that degradation of DNA and/or protein might occurr during the production of the test materials, affecting DNA and/or protein based GMO quantification.

Difficulties exist for determining what should be the most appropriate positive reference material or control. If one considers the approved GMO-line, it is likely that it does not match with the genetic make-up of the commercial variety. On the other hand, if the commercial variety is taken into account, then the exact genetic composition is simply not known.

Another aspect to consider when setting up a validation method is the choice of the most appropriate negative control. The only "true" negative control for analytical methods should be the parental organism used in the laboratory at the time of cell transformation. In other words, some of the protoplasts used in the transformation process should be used to regenerate crops for obtaining a source of negative reference material.

Finally, great biological difference may exist between the "approved variety" and the "cultivated variety". Since such biological difference may reflect great differences at the molecular level, problems may arise for calculation of quantities based on measurements of gene copy numbers. Likewise, the appropriate choice of reference materials for method development and analysis requires specific attention.

5 CONCLUSIONS

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

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

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

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

While GMO testing techniques are continuously improving with respect to accuracy, reliability and speed, it should be stressed that their performance is strongly affected by sampling strategies and processes. Sampling plans are to be thoroughly evaluated and defined for each new crop material and/or ingredient. It is therefore necessary to work towards the establishment of internationally accepted and harmonized sampling protocols such as those being developed by the Working Group "Genetically Modified Foodstuffs“ in Technical Committee CEN/TC 275 of the European Committee for Standardization (CEN, Brussels, Belgium) (Heissenberger, 2000).

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

6 ACKNOWLEDGEMENTS

We would like to thank Elke Anklam, Ferruccio Gadani, Hans Pijnenburg, and Bert Popping for their contribution to the manuscript.

7 TABLES

Table 1A. DNA isolation methods used for the detection of GMOs in plant material and plant-derived food products. PCR=Polymerase chain reaction, CTAB=cetyltrimethylammonium bromide, SDS=sodium dodecyl sulphate, +=successful PCR amplification, **-**=unsuccessfull PCR amplification (source: Anklam et al., 2002).

Method	DNA quality	DNA yield	PCR	Material	References
Wizard™ method			+	Soybean powder, maize powder	Spoth & Strauss (1999)
			+	Bruised Soya grain,lecithin	Brodmann et al. (1997)
			+	Soya leaves	Köppel et al. (1997)
	High	Low	+	Soya products (tofu, flour, lecithin)	Zimmermann et al. (1998a)
			-	Soybean oil	Pauli et al. (1998)
			+	Foodstuffs derived from soybean, corn, rice, sugar beet, tomato and wheat	Pauli et al. (2000)
CTAB method			+	Raw potato	L24.01.1-1 (1997)
			+	Raw soybeans	L23.01.22-1 (1998)
			+	Raw tomato	L25.03.01 (1999)
	High	Low	+	Soya products (tofu, flour, lecithin)	Zimmermann et al. (1998a)
			+	Foodstuffs derived from potato	Pauli et al. (2000)
CTAB method + QIAquick column			+	Soya products: meal, oil, lecithin, tofu, chocolate, etc.	Meyer & Jaccaud (1997)
			-	Soya sauce, refined soya oil	
CTAB method + Nucleon Phytopure			+	Maize, potato, soya, sugar-beet, tomato	Pietsch et al. (1997)
Nucleon Phytopure method	High	Low	+	Soya products: tofu, flour, lecithin	Zimmermann et al. (1998a)
Qiagen DNeasy method					
Chelex 100 method	Low	High	+		
Alkali method					
AlkaliX method					
ROSE method					
ROSEX method					
Dellaporta method			+	Maize grains	Ehlers et al. (1997) Dellaporta (1994)
Hexane/guanidine thiocyanate + gel filtration			+	Raw and purified, lecithin	Wurz et al. (1998)
CTAB method + High Pure PCR Template Preparation Kit (Boehringer)			+	Bt-maize in silage	Hupfer et al. (1999a)
Modified QIAamp DNA Stool Mini Kit			+	Cacao-derived products	Tengel et al. (2000)
SDS/Rnase method + Magyx silica-magnetite-based solid-phase support			+	Dried soybean, soybean flour, extruded defatted soya acid, alcohol-precipitated soya concentrate, lecithin, maize grits, seasoned corn puffs, salted corn chips	Hurst et al. (1999)

Table 1B. DNA isolation methods used for molecular marker analysis (AFLP, RAPD, PCR-RFLP) in plant material and plant-derived food products (source: Anklam et al., 2002).

Method	DNA quality	DNA yield	PCR	Material	References
NaI method			+	Single-seed *(B. oleracea L.)*	Sakamoto et al. (2000)
Modified Mettler's method for a single pollen grain			+	Single pollen (*Fagus sylvatica*)	Krabel et al. (1998)
Modified CTAB method + activated charcoal			+	Cotton, coffee, rubber tree, cassava, banana	Vroh Bi et al. (1996)
Modified CTAB method + spermine			+	Woody species: bark, dormant buds, etc.	Cheng et al. (1999)
Carlson/Qiagen (CTAB method + column)	Good	High	+	Leaves and needles: oak, elm, pine, fir, poplar, maize	Csaikl et al. (1998)
Ziegenhagen-upscaled	Good	Medium	+		
Doyle & Doyle	Medium	Medium	+		
Dellaporta/Qiagen (SDS method + column)	Medium	Low	+		
0.5 N NaOH - "grinding & use"-protocol			+	Young leaves: *A. thaliana, B. napus*, tobacco, etc.	Wang et al. (1993)
Treatment by proteinase K in SDS extraction buffer + grinding			+	Dry seeds	Hee Wan Kang et al. (1998)
Modified phenol-chloroform-EtOH-protocol			+	Dry tea	Singh et al. (1999)
Modified Nucleon PhytoPure method			+	Cured tobacco leaves (flue-cured, Burley, Oriental tobacco)	Ross et al. (1998), Rossi et al. 2000), cited in Bindler et al. (1999)
CTAB method			+		
Qiagen DNeasy Plant Mini Kit			+		Pijnenburg, (1998) cited in Gadani et al. (2000)
Method for separation and accumulation of DNA from oil			(+*)	Rapeseed oil	Hellebrand et al. (1998)

(+*) DNA from rapeseed oil could not be identified unequivocally

Table 2. Characteristics of some DNA polymerases used for PCR

	Taq/ AmpliTaq ®	Vent™	Deep-Vent™	Pfu	Tth	UITma™
Source	Thermus aquaticus	Thermo-coccus litoralis	Pyrococcus GB-D	Pyrococcus furiosus	Thermus thermophilus	Thermotoga maritima
Application	*Taq*: natural AmpliTaq: for genetic engineering	For genetic engineering	For genetic engineering	Natural	For genetic engineering	For genetic engineering
$T_{½}$ of activity at 95 ºC (min)	40	1380	400	>120	20	>50[a]
5’ to 3’ Exonuclease activity	Yes	No	No	No	Yes	No
3’ to 5’ Exonuclease activity	No	Yes	Yes	Yes	No	Yes
Processivity	50-60	?	7	?	30-40	?
Extension rate (nt/s)	75	?	>80	60	>33	?
Resulting DNA ends	3’A	>95% blunt	>95% blunt	?	3’A	blunt
MW in kDa	94	?	?	92	94	70

Table 3. Screening strategy: Generic GMO markers introduced into approved GM crops and useful for the development of a screening method (source: Hemmer, 1997).

Generic GMO markers	**Identifiable Products (total 28)**
*P-35S**	22
NOS 3'	16
P-35S, NOS 3'*	26 (or 27)
P-35S, NOS 3', E9 3', als*	28
nptII	17
P-35S, nptII*	25
P-35S, nptII, NOS 3'*	26 (or 27)
*P-NOS**	7
P-35S, P-NOS**	25

als = acetolactate synthase gene (sulfonylurea tolerance)
E9 3' = 3' sequence of small subunit of rbcS (ribuose-1,5-bisphophate carboxylase) E9 gene from pea
NOS = nopaline synthase gene
NOS 3' = nos terminator sequence from *Agrobacterium tumefaciens*
nptII = neomycin-phosphotransferase II
P-35S = cauliflower mosaic virus 35S promoter sequence
*P-35S** = P-35S including derivatives
P-NOS 3' = nos promoter sequence from *Agrobacterium tumefaciens*

Table 4A. PCR methods for detection of genetically modified maize (source: Anklam et al. 2002). Scr=screening, ID=identification

GMO product (Company)	Scr/ID	Primer	Amplicon length	Primer reference	Target gene or genetic element	Target sequence reference	References
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)	ID	*cry* IA(b)	184 bp	+	*crylA(b)* gene (from *Bacillus thuringiensis*)	Not published	Ehlers et al. (1997)
		bar	264 bp	+	*bar* gene (from *Streptomyces hygroscopicus)*	Not published	
		35S-bar	365 bp	+	*Crossborder sequence: CaMV-promoter/*bar *gene*	GenBank A18053	
		ampR	828 bp	+	*ampR* gene (from Plasmid pUC19 from *E. coli*)		
	Control	*ivr1*	226 bp	+	Exon 3 of *ivr1* gene (corn-specific single copy gene)	GenBank U16123	
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)	ID	Cry01/Cry02	1914 bp	+	*crylA(b)* gene (from *Bacillus thuringiensis*)	Koziel et al. (1993)	Hupfer et al. (1997)
	Control	TR03/TR04	137 bp	Allmann et al. (1993)	18S-rDNA (highly conserved sequence)	Allmann et al. (1993)	
Bt-maize Event 176 (Ciba Seeds, USA, Ciba-Geigy/Novartis)	ID	Cry01/Cry02	1914 bp	Hupfer et al. (1997)	*crylA(b)* gene (from *Bacillus thuringiensis*)		Hupfer et al. (1998)
		Cry03/Cry04	211 bp	+	Crossborder sequence: CDPK-promoter/*crylA(b)* gene		
	Control	Ivr1-F/Ivr1-R	226 bp	Ehlers et al. (1997)	Exon 3 of *ivr1* gene: (corn-specific single copy gene)		
MaisGard MON810 (Monsanto, USA)	ID (nested PCR)	mg1/mg2	401 bp	+	Crossborder sequence: CaMV 35S promoter/intron 1 of *hsp70*	Monsanto (1995), APHIS petition No. 95-093-01p	Zimmermann et al. (1998b)
		mg3/mg4	149 bp	+	Crossborder sequence: CaMV 35S promoter/exon 1 of *hsp70*		
	Control (nested PCR)	hm1/hm2	175 bp	+	*HMG* gene (highly conserved sequence)	Krech (1997)	
		hm3/hm4	149 bp	+			
Bt11 (Novartis Seeds, USA)	ID	IV01/CR01	431 bp	+	Crossborder sequence: intron 6 of *adh1*-1S gene/*crylA(b)* gene	*crylA(b):* Koziel et al (1997), US Patent 5,625,136 *pat:* Wohlleben et al. (1988) CaMV: Sanders et al. (1997) CDPK: Estruch et al. (1994) PEPC: Hudspeth & Grula (1989) *hsp70:* Rochester et al. (1986) *adh1*-S1: Freeling & Bennet (1985)	Matsuoka et al. (2000)
Bt-maize Event 176 (Novartis Seeds, USA)		PE01/CR01	619 bp	+	Crossborder sequence : PEPC promoter/*crylA(b)* gene		
MON810 (Monsanto, USA)		HS01/CR01	194 bp	+	Crossborder sequence: *hsp70* intron 1/*crylA(b)* gene		
LIBERTY T14 or T25 (Hoechst Schering AgrEvo, Germany)		GM03/PA01	231 bp	GM03 by Köppel et al. (1997)	Crossborder sequence: CaMV 35S promoter/*pat* gene		
Bt11, Event 176, MON810, LIBERTY-maize	Scr	CM01/02	220 bp	Matsuoka et al. (2000)	CaMV 35S promoter		
	Control	ZE01/02	329 bp	Studer et al. (1997)	*ze1* gene (corn-specific single-copy gene)		
Maximizer maize (Plant Genetic Systems, Ciba Seeds)	ID (nested PCR)	CRY1A1/CRY1A2	420 bp		*crylA(b)* gene (from *Bacillus thuringiensis)*	Koziel et al (1997), US Patent 5,625,136	Studer et al. (1997)
		CRY1A3/CRY1A4	189 bp				
	Control (nested PCR)	ZEIN1/ZEIN2	485 bp		*ze1* gene (corn-specific single-copy gene)	Kirihara et al. (1988)	
		ZEIN3/ZEIN4	277 bp				
GM maize	ID	Forward/reverse	540 bp		Crossborder sequence: CaMV 35S promoter/*dhfr* gene	Golovkin et al. (1993)	Hemmer (1997)
			840 bp				
SeedLInk maize (Plant Genetic Systems)	ID	Forward/reverse	160 bp		barnase	Petition from Plant Genetic Systems for SeedLink (1995), APHIS Petition 95-228-01p	
			235 bp		barstar		
Bt-maize Event 176	ID	btsyn f1/btsyn r1	151 bp		*crylA(b)* gene (from *Bacillus thuringiensis)*		DMIF-GEN, Final Report (1999)
GM maize		bar-af1/bar-ar	278 bp		*bar/pat* genes from *Streptomyces viridochromgenes s. hygroscopicus*		
Bt-maize Bt-176		CRYFZ 1/CRYFZ 2	150 bp		*crylA(b)* gene (from *Bacillus thuringiensis)*		

Table 4B. PCR methods for detection of genetically modified soybean (source: Anklam et al. 2002). Scr=screening, ID=identification

GMO product (Company)	Scr/ID	Primer	Amplicon length	Primer reference	Target gene or genetic element	Target sequence reference	References
Roundup Ready soybean (Monsanto, USA) dried soybean, soybean flour, extruded defatted soya acid, alcohol-precipitated soya concentrate	Scr	35S-1/-2	195 bp	Pietsch et al. (1997)	CaMV 35S promoter		Hurst et al. (1999)
	ID (multiplex PCR)	SPA/SPB	320 bp	+	Crossborder sequence: CaMV 35S promoter/EPSPS gene		
	control	LE1/LE2	407 bp	+	*le1* gene (soya-specific single copy gene)	Vodkin et al. (1983)	
Roundup Ready soya lecithin (Monsanto, USA)	ID (multiplex PCR)	SPA/SPC	120 bp	+	Crossborder sequence: CaMV 35S promoter/CTP sequence (CTP from *Petunia hybrida*)		
	control	LE5/LE6	180 bp	+	*le1* gene (soya-specific single copy gene)	Vodkin et al. (1983)	
Roundup Ready soybean (Monsanto, USA)	Scr	35S-1/-2	195 bp	+	CaMV 35S promoter	Kay et al. (1987), Science 236: 1299-1302	Pietsch et al. (1997)
		NOS-1/NOS-2	180 bp	+	NOS-terminator (from *Agrobacterium tumefaciens*)		
	control	plant-1/-2	500-600 bp	+	Non-coding region from chloroplast genome	Taberlet et al. (1991)	
Raw and fractionised Roundup Ready lecithins (Monsanto, USA)	ID	p35s-af2/petu-ar1	172 bp	Wurz & Willmund (1997)	Crossborder sequence: CaMV 35S promoter/CTP sequence		Wurz et al. (1998)
	control	sole-af1/2	145 bp	+	*le1* gene (soya-specific single copy gene)	Vodkin et al. (1983)	
Roundup Ready soybean (Monsanto, USA)	ID	p35s-f2/petu-r1	172 bp	+	Crossborder sequence: CaMV 35S promoter/CTP sequence	Padgette et al. (1995)	Wurz & Willmund (1997)
	control				*le1* gene (soya-specific single copy gene)		
Glyphosate-tolerant soybean seeds, Code No. 9396 (Pioneer Hi-Bred International, Inc. Iowa, USA)	ID (nested PCR)	GM05/GM09	447 bp	+	Crossborder sequence: CaMV 35S promoter/EPSPS gene	Monsanto patent WO 92/04449 Padgette et al. (1995)	Meyer & Jaccaud (1997)
		GM07/GM08	169 bp	+	Crossborder sequence: CaMV 35S promoter/CTP sequence		
	Control (nested PCR)	GM01/02	414 bp	Meyer et al. (1996)	*le1* gene (soya-specific single copy gene)		
		GM03/04	118 bp				
Glycine max soybean (non-GMO) meat products	ID	GM01/GM02	414 bp	+	*le1* gene (soya-specific single copy gene)	Vodkin et al. (1983)	Meyer et al. (1996)
		GM03/04	118 bp	+			
	control	TR03/TR04	137 bp	Allmann et al. (1993)	18S-rDNA (highly conserved sequence)	Allmann et al. (1993)	
Roundup Ready soybean (Monsanto, USA)	ID (nested PCR)	RR01/RR02	509 bp	+	Crossborder sequence: *CP4 EPSPS* gene/CaMV 35S promoter		Köppel et al. (1997)
		RR05/RR04	180 bp	+	Crossborder sequence: *CP4 EPSPS* gene/CTP sequence		
	Control (nested PCR)	GM01/02	414 bp	Meyer et al. (1996)	*le1* gene (soya-specific single copy gene)		
		GM03/04	118 bp				
		TR03/04	137 bp	Allmann et al. (1993)	18S-rDNA (highly conserved sequence)		
Roundup Ready soybean (Monsanto, USA)	ID	Forward/reverse	475 bp		NptII gene	Petition from Monsanto for herbicide-tolerant soybean (1993), APHIS petition 93-258-01p	Hemmer (1997)
Roundup Ready soybean (Monsanto, USA)	ID	CAM/CTP	110 bp		Crossborder sequence: CaMV 35S promoter/CTP sequence		DMIF-GEN, Final Report (1999)
		EPS 1/NOS a	147 bp		Crossborder sequence: *CP4 EPSPS* gene/*nos* terminator		
	Control	LEC 1/LEC 2	164 bp		*le1* gene (soya-specific single copy gene)		

Table 4C. PCR methods for detection of genetically modified tomato, potato, sugarbeet, cotton, papaya, alfalfa, and tobacco (source: Anklam et al. 2002). Scr=screening, ID=identification

GMO product (Company)	Scr/ID	Primer	Amplicon length	Primer reference	Target gene or genetic element	Target sequence reference	References
Flavr Savr tomato (Calgene Inc., USA) Changin potato (Station fédérale de recherche en production végétale de Changin, Switzerland), Glyphosinate-resistent sugarbeet (AgrEvo, Germany)	Scr	35S-1/35S-2	195 bp (+ 390 bp in tomato)	+	CaMV 35S promoter	Kay et al. (1987)	Pietsch et al. (1997)
		Tn5-1/Tn5-2	173 bp	Identical to corresponding primer pair of Meyer (1995)	nptII gene (from transposon Tn5)	Beck et al. (1982)	
	Control	plant-1/-2	500-600 bp	+	Non-coding region from chloroplast genome	Taberlet et al. (1991)	
Changin potato (Station fédérale de recherche en production végétale de Changin, Switzerland) B33-Invertase potato (IGF, Germany)	Scr	NOS-1/NOS-2	180 bp	+	NOS-terminator (from Agrobacterium tumefaciens)	Kay et al. (1987)	
	Control	plant-1/-2	500-600 bp	+	Non-coding region from chloroplast genome	Taberlet et al. (1991)	
Flavr Savr tomato (Calgene Inc., USA)	ID	PCR1/FS01	427 bp	PCR1 by Jongedijk et al. (1992)	Crossborder sequence: CaMV 35S promoter/PG gene	FS01 according to Sheehy et al. (1988); Smith et al. (1988)	Meyer (1995)
	Scr	Tn5-1/ -2	173 bp	+	nptII gene (from transposon Tn5 on pBIN19 from A.tumefaciens)	Padegimas et al. (1993)	
	Control	TR03/04	137 bp	Allmann et al. (1993)	18S-rDNA (highly conserved sequence)	Allmann et al. (1993)	
GM Desiree potato GM Rustica potato (gbss-antisense constructs)	Scr (nested PCR)	Tn5-1/-2	173 bp	Meyer (1995)	nptII gene	T-DNA: An (1995) gbss: Kuipers et al. (1992); Hergersberg (1988) B33: Rocha-Sosa et al. (1989)	Hassan-Hauser et al. (1998)
		T-ocd-1/T-nos-2	432 bp	+	T-DNA (from modified pBIN19/near right border)		
		T-gene-III-1/T-lacI-2	405 bp	+	T-DNA (from modified pBIN19/near right border)		
		T-lacZ-1/T-M13-2	409 bp	+	T-DNA (from modified pBIN19/near left border)		
	ID (nested PCR)	T-geneIII-1/T-M13-2	3800 bp	+	Crossborder sequence: T-DNA/B33/link/gbss-AS/T-DNA		
		B33-1/gbss-as-2	580 bp instead of 530 bp	+	Crossborder sequence: B33/link/gbss-AS (incl. confidential adapter seq.)		
	Control	universal –1/-2	550 bp	+	Non-coding region from chloroplast genome	Taberlet et al. (1991)	
GM tomato (Zeneca)	ID	Forward/reverse	472 bp		Antisense PG/CaMV 35S promoter	Petition from Zeneca for genetically modified tomatoes (1995), APHIS Petition 95-290-01p	Hemmer (1997)
			478 bp		Sense PG/CaMV 35S promoter		
			943 bp		NptII gene /nos promoter		
			658 bp		Gene IIIA/nos terminator		
			890 bp		CaMV 35S promoter/nos terminator		
			401 bp		NptII gene/ocd gene		
			660 bp		Ocd gene/gene IIIA		
			180 (380) bp		PG gene (polygalacturonase)		

GM potato	ID	Forward/reverse	502 bp		CaMV 35S promoter/PVX cp gene	Jongedijk et al. (1992)	Hemmer (1997)
GM cotton (DuPont)	ID	Forward/reverse	642 bp		Als gene	Petition from DuPont for genetically modified cotton (1995), APHIS Petition 95-256-01p	
GM papaya	ID	Forward/reverse	674 bp		Gus gene	Yang et al. (1996)	
GM alfalfa	ID	Forward/reverse	1097 bp		Gus gene	Blake et al. (1991)	
			785 bp		NptII gene		
GM tobacco	ID	Forward/reverse	880 bp		P-TA29 promoter	Kriete et al. (1996)	
GM potato (B33-INV)	ID	B1/B2	839 bp		AphIV gene (hygromycinphosphotransferase)		LMBG L24.01-1 (1997)
	Control	A1/A2	550 bp		Chloroplast tRNA gene		
Nema 282F tomato (Zeneca)	Control	PG34L/PG34R	383 bp		PG gene (sense)	Gierson et al. (1986) Smith et al. (1990)	LMBG L25.03.01 (1999)
	Scr		180 bp		PG gene (antisense-construct)		
	ID	PG34L/B1	351 bp		Crossborder sequence: pg gene/nos terminator		

Abbreviations used in Table 4:

CaMV	Cauliflower mosaic virus
EPSPS	5-enol-pyruvylshikimate-3-phosphate synthase
CTP	chloroplast transit peptide
CDPK	calcium-dependent protein kinase
crylA(b)	crystal protein gene
bar	phosphinothricin (= Basta) resistence gene
ampR	ampicillin resistence gene
ivr1	invertase gene
hsp70	heat shock protein gene
pat	phosphinothricin acetyl transferase
PEPC	phosphoenolpyruvate carboxylase
adh1-S1	alcohol dehydrogenase 1 gene
ze1	zein gene
le1	lectin gene
HMG	high-mobility-group protein gene
nptII	neomycin phosphotransferase II gene
"Flavr Savr"	polygalacturonase gene from tomato in antisense
NOS	nopaline synthase
gbss	granule bound starch synthase gene
B33	tuber-specific patatin gene promoter
IGF	Institut für Genbiologische Forschung

Table 5. Calculations of genome copies in typical PCR working solutions.

	1C value (in pg)	*1	*2	*3
Glycine max	1.25	$8\ 10^4$	80	0.00125%
Zea Mays	2.6	$3.7\ 10^4$	37	0.0026%
Triticum aestivum	17.352	$5.8\ 10^3$	5	0.0173%

*1: Number of genome copies in a solution containing 100 ng DNA
*2: Number of "GMO" genome copies in a 100 ng DNA solution, that is made of 0.1% GMO
*3: Theoretical limit that cannot be gone beyond for a 100 ng DNA solution

Table 6. Expected probability of GMO copies in a sample of 1000ng of DNA, at a nominal concentration of 0.005%. While the most probable number of copies would be 18, there is a 0.57% chance that 8 or fewer copies will be present.

Number of GM copies	Cumulative Binomial probability	Frequency	Actual concentration
8	0.57%	0.3%	0.002%
9	1.27%	0.7%	0.002%
10	2.55%	1.3%	0.003%
11	4.69%	2.1%	0.003%
12	7.96%	3.3%	0.003%
13	12.57%	4.6%	0.004%
14	18.61%	6.0%	0.004%
15	26.01%	7.4%	0.004%
16	34.49%	8.5%	0.004%
17	43.64%	9.2%	0.005%
18	**52.97%**	**9.3%**	**0.005%**
19	61.98%	9.0%	0.005%
20	70.24%	8.3%	0.005%
21	77.46%	7.2%	0.006%
22	83.48%	6.0%	0.006%
23	88.29%	4.8%	0.006%
24	91.96%	3.7%	0.007%
25	94.66%	2.7%	0.007%
26	96.56%	1.9%	0.007%

Table 7. Parameters determined through performance and validation studies (source: Anklam et al. in press).

Term	**Description**
Specificity	The probability of obtaining a negative result, given that there is no analyte present
Linearity	Proportionality of the signal to the amount of reference material, demonstrated by the calculation of a regression line with the adequate statistical method.
Range	Range of analyte concentrations over which the method is considered to perform in a linear manner
Accuracy	Comparison with a reference procedure or the recovery by the assay of a known added amount of analyte
Precision	Intra-laboratory variation (repeatability) and inter-laboratory variation (reproducibility). The recommended data include standard deviation, relative standard deviation (coefficient of variation) and confidence interval.
Detection Limit	Limit of Detection (LOD) Minimum level at which the analyte can reliably be detected
Quantitation limit	Limit of Quantitation (LOQ): is determined by analysis of known samples and establishes the minimum level at which the analyte can be quantified.
Robustness	Stability of the method; reliability of the method with respect to deliberate variations in the method parameters.

Table 8. Qualitative PCR detection methods for screening submitted to collaborative studies (source: Anklam et al. in press).

Matrix	Number of Laboratories (excluded)	Samples	Performance	Coordinator (Reference)
Soybean raw material and lecithin	6	0%, 0.1%, 1%, 100%	~93% overall accuracy for 35S 80 results (2 false positives, 4 false negatives) ~90% overall accuracy for NOS 80 results (8 false negatives)	Official Swiss method, (Brodmann et al., 1997)
Flour of soybeans and maize	29 (7 for maize 4 for soybean)	0%, 0.1%, 0.5%, 2%	98.8% specificity for 35S in maize 93.5% sensitivity for 35S in maize 97.9% specificity for 35S in soya 98.4% sensitivity for 35S in soya 100% specificity for NOS in soya 96.7% sensitivity for NOS in soya	JRC, (Lipp et al., 1999)
Processed foods with soybeans and/or maize	23 (6)	0%, 2%, 100%	96.1% specificity for 35S 98.1% sensitivity for 35S 98.2% specificity for NOS 97.9% sensitivity for NOS	JRC, (Lipp et al., 2001)

Table 9. Qualitative PCR methods for specific identification submitted to collaborative studies (source: Anklam et al. in press).

Matrix	Number of Laboratories (excluded)	Samples	Performance	Coordinator (Reference)
raw potato	18	0%, 100%	~98% overall accuracy, 163 samples (3 false negative, 1 false positive)	BgVV, (LMBG 24.01-1, 1997)
Raw soybeans	25 (3)	0%, 100%	100% overall accuracy, 110 samples	BgVV, (LMBG 23.01.22, 1998)
Raw tomato	19 (1)	0%, 100%	100% overall accuracy, 90 samples	BgVV, (LMBG 25.03.01, 1999)
Soy meal	16 (4)	0%, 0.1%, 0.5%, 2%	100% overall accuracy, 96 samples	DMIF-GEN, 1999
Maize flour	18 (3)	0%, 0.1%, 0.5%, 2%	~97% overall accuracy, 75 samples (1 false negative, 1 false positive)	DMIF-GEN, 1999
Processed foods with soya/maize	15	0%, 2%, 100% 1% meals	~89% overall accuracy, 108 samples (11 false positive, 1 false negative)	BgVV, (EU Tender Report, 2000)
Soybean raw material and lecithin	6	0%, 0.1%, 1%, 100%	~96% overall accuracy for RRS, 88 results (4 false negative)	Official Swiss method, (Brodmann et al., 1997)

Table 10. Quantitative PCR methods submitted to collaborative studies (source: Anklam et al. in press).

Matrix	Number of Laboratories (excluded)	Samples	Type of Assay	Performance	Cooordinator (Reference)
Soybean flour and processed foods	12	0% to 2%	QC-PCR/semi-quantitative	Test if sample contains 0%, <0.5%, between 0.5% and 2%, or >2% GMO 0.5% RRS: 9% CV 2% RRS: 2% CV 0.810 contingency coefficient for RRS specific versus 35S-promoter specific QC-PCR	KLZ, (Hübner et al., 1999)
Maize meals	19	0%, 0.1%, 0.5%, 1.7%, 2%	QC-PCR/semi-quantitative	Test if sample contains >1%, <1% or 0% GMO: 0% samples ~86.8% correct results 0.1% samples ~97.4% correct results 0.5% samples ~63.2% correct results 1.0% samples ~81.6% correct results 2.0% samples ~94.7% correct results	DMIF-GEN, 1999
Soybean flour	13 (2)	0%, 0.1%, 1%, 2%	PCR-ELISA/semi-quantitative	0% GMO samples 91% correct results 0.1% GMO samples <29% correct results (0.1% GMO was correctly classified after comparison with 0.5% positive control) 1.0% GMO samples 100% correct results 2.0% GMO samples 100% correct results	DMIF-GEN, 1999
Soybean meals and texturized vegetable protein (TVP)	28 14 (ABI 7700) 6 (2) (ABI 5700) 12 (5) (LightCycler)	0.1%, 0.5%, 1%, 2%, 5% 2% TVP	Realtime-PCR/ Quantification	23-28% coefficient of variation for ABI 7700 30-82% coefficient of variation for ABI 5700 16-41% coefficient of variation for LightCycler	BgVV, (EU Tender Report, 2000)
Maize and soybean meals	23 (9 for maize) (10 for soybean)	0%, 0.1%, 0.5%, 1%, 2%, 5%	Double Competitive PCR/semi-quantitative	For 0%, 0.1%, 0.5%, 1.0% GM-maize samples 73-85% correct results For 2% GM-maize samples 46% correct results For 5% GM-maize samples 23% correct results For 0%, 0.1%, 0.5%, 1%, 2%, 5% GM-soybean samples 70-75% correct results	JRC, (Van den Eede et al., 2000)

Table11. Immunoassay methods submitted to collaborative studies (source: Anklam et al. in press).

Matrix	Number of Laboratories (excluded)	Samples	Performance	Co-ordinator [Reference]
Dried soybean powder	38 (2-4)	0%, 0.5, 1%, 2%	Semi-quantitative Test if samples contains >2%, <2% GMO (1.25% reference standard) 2% GMO samples 99% correct results 1% GMO samples 95% correct results 0.1% GMO samples 100% correct results 0% GMO samples 100% correct results Quantitative LOD 0.35% RSD_r: 7.3% to 12.4% RSD_R: 9.3% to 16.6%	JRC, (Lipp and Anklam, 2000)
Maize flour	40	0%, 0.3%, 0.5%, 0.75%, 1%, 2%	RSD_R <23%	AACC, (Stave et al., 2001)

Table 12. Certified Reference Materials For GMO Detection

CRMs of genetically modified roundup ready TM soya beans (IRMM-410)

SB - 0:	0 % GMO (1 g units)
SB - 0.1:	0.1 % GMO (1 g units)
SB - 0.5:	0.5 % GMO (1 g units)
SB - 1:	1 % GMO (1 g units)
SB - 2:	2 % GMO (1 g units)
SB - 5:	5 % GMO (1 g units)

CRMs of genetically modified Bt-176 maize (IRMM-411)

MZ - 0:	0 % GMO (2 g units)
MZ - 0.1:	0.1 % GMO (1 g units)
MZ - 0.5:	0.5 % GMO (1 g units)
MZ - 2:	2 % GMO (1 g units)

MZ – 1:	1 % GMO (1 g units)
MZ – 5:	5 % GMO (1 g units)

CRMs of genetically modified Bt-11 maize (IRMM-412)

MA - 0:	0 % GMO (2 g units)
MA - 1:	1 % GMO (1 g units)
MA - 2:	2 % GMO (1 g units)

CRM of genetically modified MON810 maize (IRMM-413)

PMON 810 - 0:	0 % GMO (2 g units)
PMON 810 – 0.1	0.1 % GMO (1 g units)
PMON 810 – 0.5	0.5% GMO (1 g units)
PMON 810 – 1	1% GMO (1 g units)
PMON 810 – 2	2% GMO (1 g units)
PMON 810 - 5	5% GMO (1 g units)

8 FIGURES

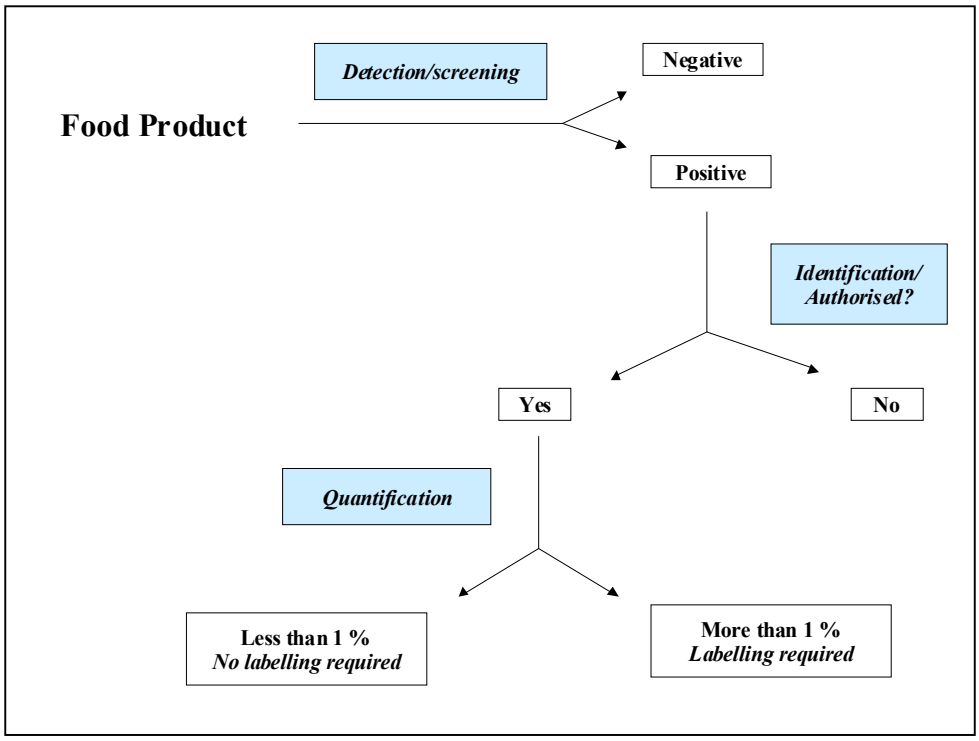

Figure 1

Operational procedures for detection, identification and quantification of GMOs in compliance with the labelling regulation in the European Union (source: Anklam et al. 2002).

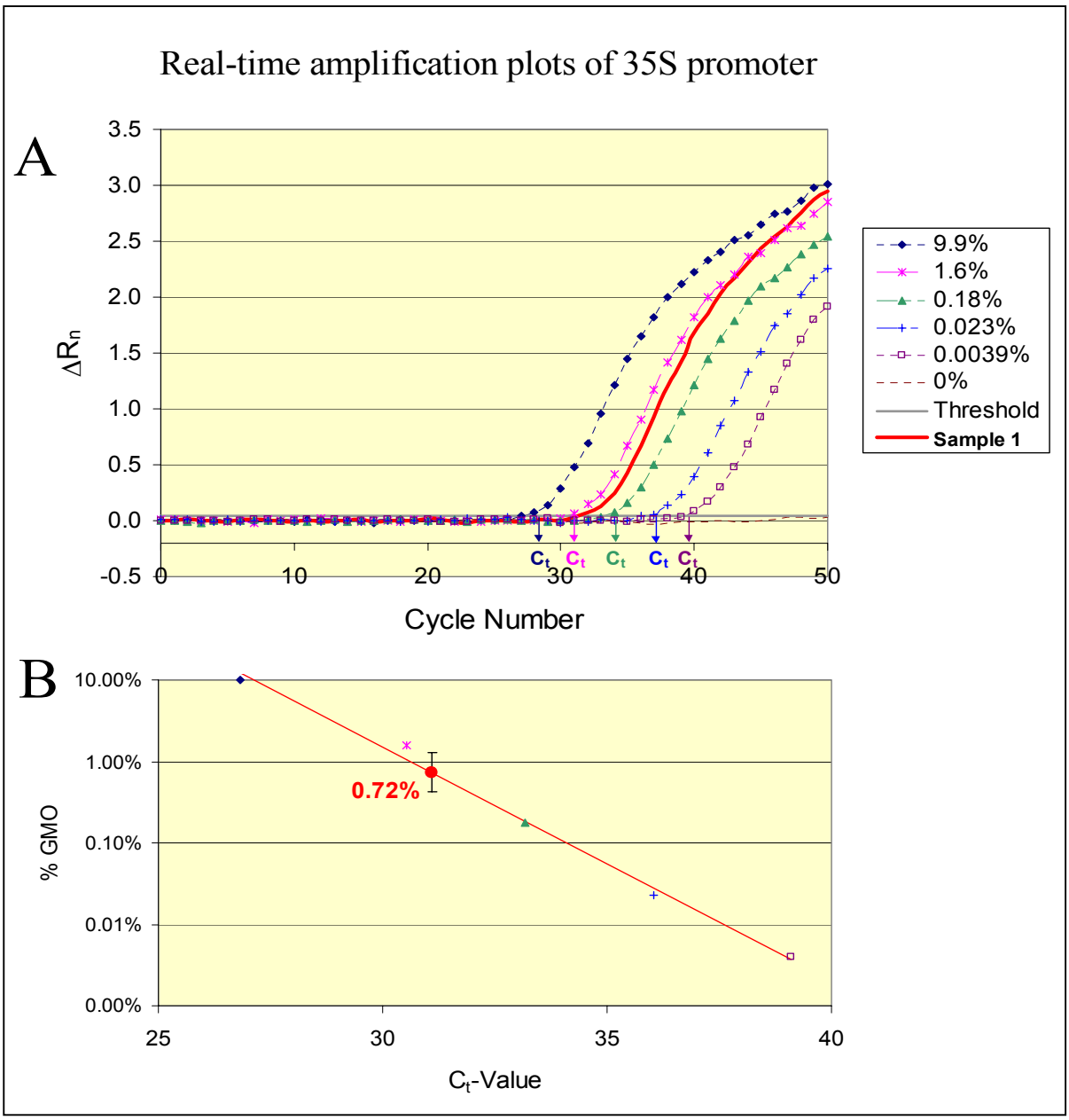

Figure 2

Real time PCR (source: Anklam et al. 2002).

A) Diagram showing the accumulation of the target analyte 35S promoter at six different ratios of GMO/non-GMO material (% w/w). PCR product formation is visualized in real time by taking fluorescence measurements (ΔR_n) at each cycle. The initial template concentration determination is based on the threshold cycle (C_t), i.e. the PCR cycle at which fluorescence is first detected statistically significant above background. C_t is inversely proportional to the log of the number of target copies present in the sample.

B) Linear regression diagram showing the logaritmic relation between the GMO/non-GMO ratios and the Ct values.

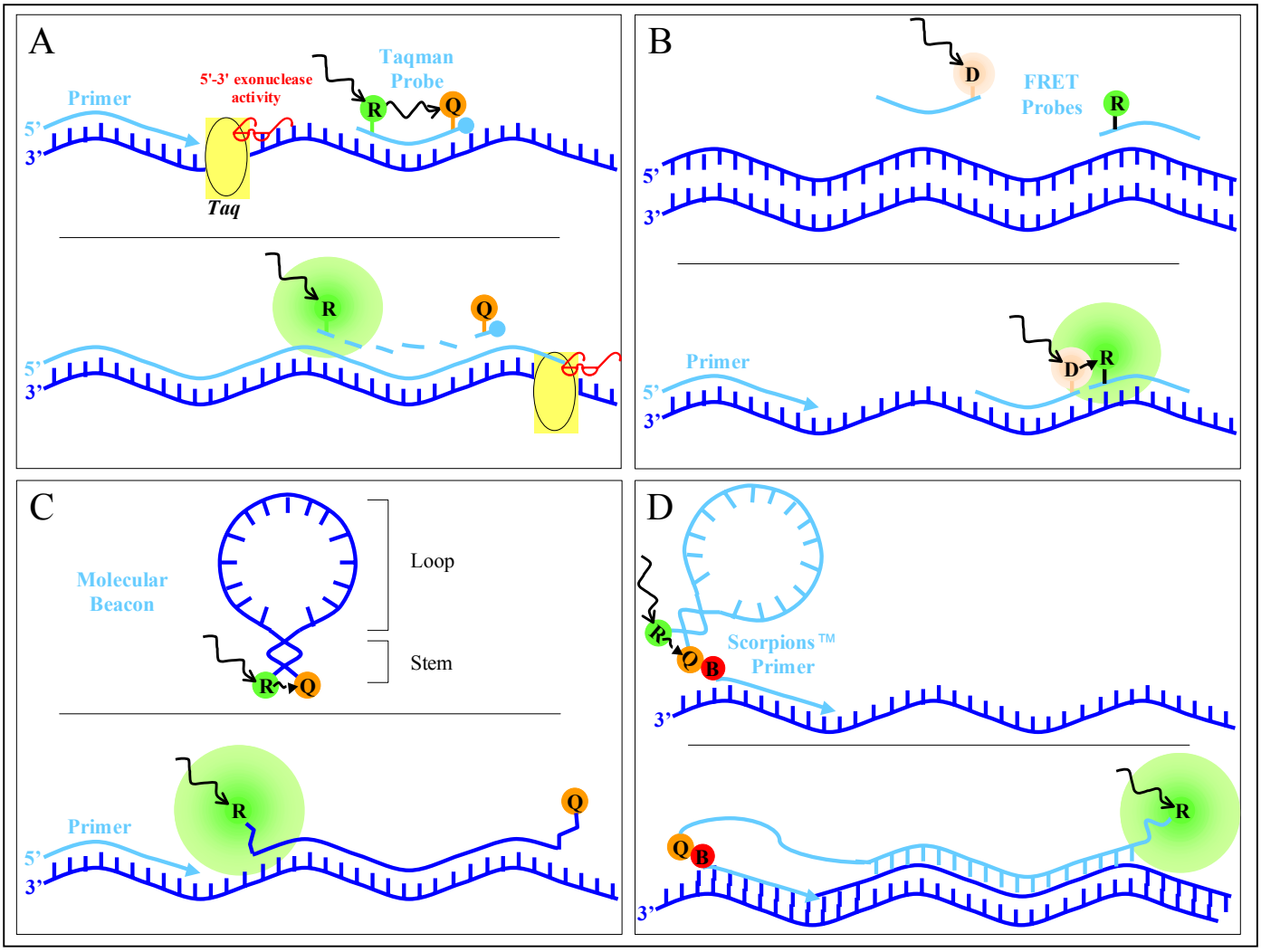

Figure 3

Different techniques to monitor the PCR product formation in real time using amplicon-specific probes.
A) Taqman Probes (see text); **B**) FRET probes: the 3'-end of one probe is labeled with a donor fluorophor, while the 5'-end of an adjacent probe is labeled with an acceptor fluorophor. In the annealing phase of each PCR cycle, the probes will hybridize on the target and the FRET phenomenon generates a measurable fluorescent signal. **C**) Molecular Beacons: hairpin-shaped oligos with a loop sequence complementary to part of the target sequence and flanked by two arms that anneal to form a short (5-7 base pair) stem. One of the arms is labeled with a reporter dye, while the other is labeled with a quencher dye. When free in solution the stem is intact and the proximity of the quencher to the reporter allow efficient quenching of the reporter dye. In the annealing phase of each PCR cycle, the loop of the Molecular Beacon hybridizes with the target and the quencher is separated from the reporter. In this situation the fluorescence of the reporter dye will be measurable. **D**) Scorpions™ primers consist of a PCR primer with the 5' end linked to a Molecular Beacon-type molecule through a "blocker". During PCR the Scorpions™ primer is extended and the specific probe sequence of the linked Molecular Beacon is able to bind to its complement within the same strand of DNA. As with a Molecular Beacons, this hybridization event opens the hairpin loop so that the reporter is not longer quenched and an increase in signal can be observed. The "blocker" between the primer and the Molecular Beacon prevents read-though of the Molecular Beacon part, which would lead to opening of the hairpin loop in the absence of the specific target sequence probes.

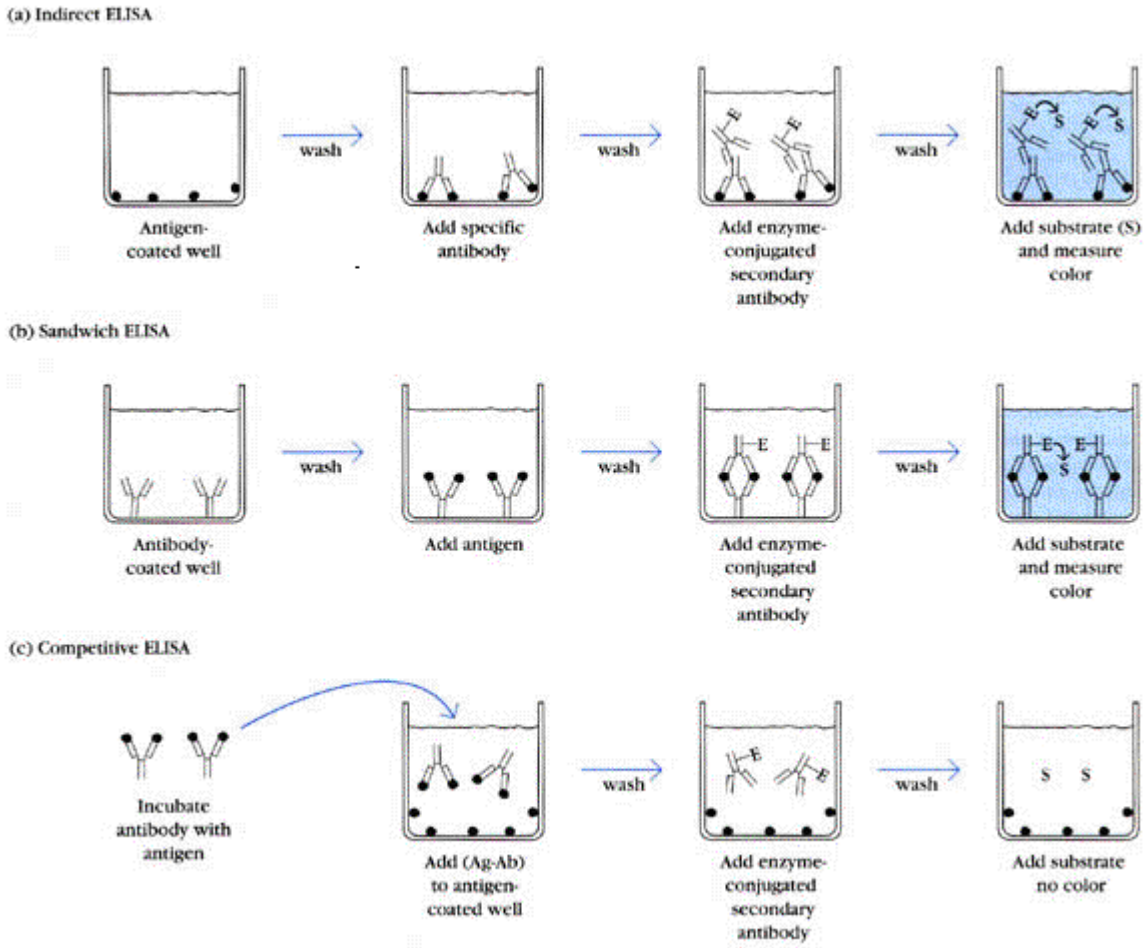

Figure 4

Different strategies to perform an ELISA Test

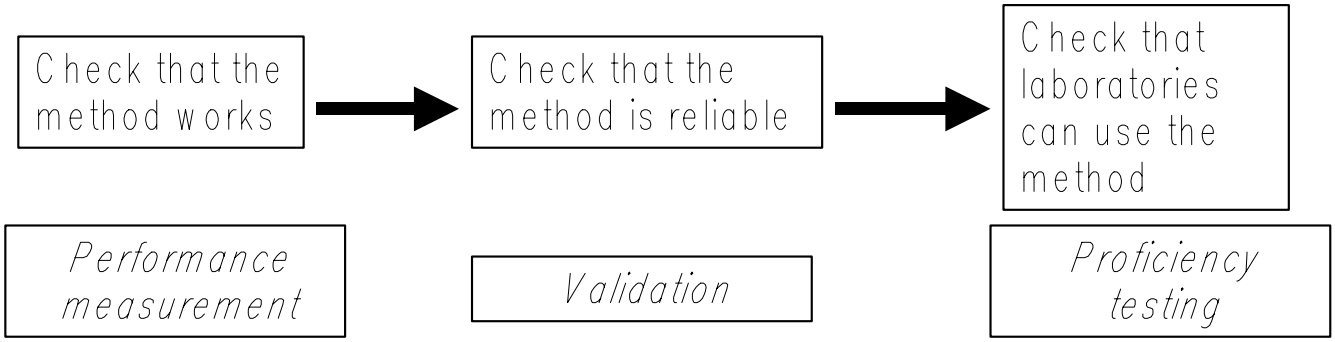

Figure 5

Performance measurement, validation, and proficiency testing processes (source: Anklam et al. in press).

9 REFERENCES

Association Française de Normalisation (AFNOR) (2000), Foodstuffs – Detection and quantification of genetically modified vegetal organisms and derived products – Part 1 – Guidelines and requirements. Association Française de Normalisation, Paris, France

Allmann M, Candrian U, Höfelein C, Lüthy J (1993), Z Lebensm Unters Forsch 196: 248–251

Ambrus A (1999) FAO-IEAA-AOAC-IUPAC International Workshop on Principles and Practices of Method Validation, Budapest

An G (1995) Binary Ti plasmid vectors. In: Gartland KMA, Davey MR (eds) Agrobacterium protocols, vol 44. In: Walker JM (series ed) Methods in molecular biology. Humana Press, Totowa, NJ, pp 47–48

Anklam E (1999) Anal Chim Acta 393: 177–179

Anklam E, Gadani F, Heinze P, Pijnenburg H, Van den Eede G (2002) Eur Food Res Technol 214: 3-26

Anklam E, Heinze P, Kay S, Van den Eede G, Popping B (in press) J. AOAC Int

Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H (1982), Gene 19: 327–336

Belgrader P, Benett W, Hadley D, Richards J, Statton P, Mariella R Jr, Milanovich F (1999) Science 284: 449–450

Belgrader P, Smith JK, Weedn VW, Northrup MA (1998) J Forensic Sci 43: 315–319

Bennett MD, Cox AV, Leitch IJ (2000) Angiosperm DNA *C*-Values database. http://www.rbgkew.org.uk/cval/database1.html.

Berger B, von Holst C (2001) Pesticides residues in products of plant origin in the European Union: sampling strategy and results from the co-ordinated EU monitoring programme in 1996 and 1997. In: Environ Sci Pollut Res, *in print*

Berkenkamp S, Kirpekar F, Hillenkamp F (1998) Science 281: 260–262

Bernard PS, Pritham H, Wittwer CT (1999) Anal Biochem 273: 221–228

Bindler G, Dorlhac de Borne F, Gadani F, Gregg E, Guo Z, Klus H, Maunders M, Mueller L, Negishi H, Pijnenburg H, Ward M, Zuber J (1999) Report of the task force genetically modified tobacco: detection methods. CORESTA Bulletin 1999-4, Cooperation Centre for Scientific Research Relative to Tobacco, Paris, France, pp 77–148

Blake NK, Ditterline RL, Stout RG (1991) Crop Science 31: 1686–1688

Bonnet G, Tyagi S, Libchaber A, Kramer FR (1999) Proc Natl Acad Sci USA 96: 6171–6176

Brett GM, Chamber SJ, Huang L, Morgan MRA (1999) Food Control 10: 401–406

Brodmann P, Eugster A, Hübner P, Meyer R, Pauli U, Vögeli U, Lüthy J (1997) Mitt Geb Lebensm Hyg 88: 722–731

Byrdwell WC, Neff WE (1996) J Liq Chromatogr Relat Technol 19(15): 2203–2225

Cayouette M, Sucharczuk A, Moores J, Tyagi S, Kramer FR (1999) Strategies Newsletter Special Issue "Sentinel Molecular Beacons" 12(3): 85–88

Cheng FS, Brown SK, Weeden NF (1999) Hortscience 32(5): 921–922

Chergui M (2000) Femtochemistry Chimia 54: 83–88

Clark M F, Adams AN (1977) Journal of General Virology 34: 475–483

Cochran WG (1977) Sampling Techniques, 428 pp, John Wiley and Sons (WIE); ISBN: 047116240X

Cooke R (1999) Science and Technology 27: 669–680

Csaikl UM, Bastian H, Brettschneider R, Gauch S, Meir A, Schauerte M, Scholz F, Sperisen C, Vornam B, Ziegenhagen B (1998) Plant Molecular Biology Reporter 16: 69–86

DellaPorta S (1994) Plant DNA miniprep and microprep: versions 2.1–2.3. In: Freeling M, Walbot V (eds) The Maize Handbook. Springer, Berlin Heidelberg New York, pp 522–525

DMIF-GEN (1999) Development of methods to identify foods produced by means of genetic engineering. EU-Project SMT4-CT96-2072. DMIF-GEN Final Report, 15 December 1999

Dumbroff EB, Gepstein S (1993) Immunological methods for assessing protein expression in plants. In: Glick BR, Thompson JE (eds) Methods in plant molecular biology and biotechnology. CRC Press LCC, Washington New York London, pp 207–223

DuPont (1995) Cotton / HT/Sulfonylurea tolerant. APHIS petition No. 95-256-01p. Biotechnology Permits, USDA, APHIS, BBEP, Riverdale, MD 20737

Ehlers B, Strauch E, Goltz M, Kubsch D, Wagner H, Maidhof H, Bendiek J, Appel B, Buhk HJ (1997) Bundesgesundheitsblatt 40: 118–121

EMEA-guideline CPMP/ICH/281/95 Topic Q2B (1995) http://www.eudra.org/humandocs/humans/ICH.htm

EMEA-guideline CPMP/ICH/381/95 Topic Q2A (1995) http://www.eudra.org/humandocs/humans/ICH.htm

Estruch JJ, Kadwell S, Merlin E, Crossland L (1994) Proc Natl Acad Sci USA 91(19): 8837–8841

European Commission (1990) Council Directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms, OJ No L117, 8.5.1990, pp 15–27 or Internet (1999) http://europa.eu.int/eur-lex/en/lif/dat/1990/en_390L0220.html

European Commission (1997) CommissionRegulation (EC) No 258/97 of the European Parliament and of the Council of 27 Juanuary 1997 concerning novel foods and novel food ingredients, *Official Journal* L 043, 14/02/1997 p 0001-0007

European Commission (1998) Council Regulation (EC) No 1139/98 of 26 May 1998 concerning the compulsory indication of the labelling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in Directive 79/112/EEC, OJ No L159, 3.6.1998, pp 4–7 or Internet (1999) http://europa.eu.int/eur-lex/en/lif/dat/1998/en_398R1139.html

European Commission (2000) Commission Regulation (EC) No 49/2000 of 10 January 2000 amending Council Regulation (EC) No 1139/98 concerning the compulsory indication of the labelling of certain foodstuffs produced from genetically modified organisms of particulars other than those provided for in Directive 79/112/EEC, OJ No L006, 11.1.2000, pp 13–14 or Internet (2000) http://europa.eu.int/eur-lex/en/lif/dat/2000/en_300R0049.html

European Commission (2000) Commission Regulation (EC) No 50/2000 of 10 January 2000 on the labelling of foodstuffs and food ingredients containing additives and flavourings that have been genetically modified or have been produced from genetically modified organisms, OJ No L006, 11.1.2000, pp 15–17 or Internet (2000) http://europa.eu.int/eur-lex/en/lif/dat/2000/en_300R0050.html

EU Tender Report (2000) Development of qualitative as well as quantitative detection methods to identify a genetic modification in soybean and maize products Report of the EU tender N. XXIV/9898A3/001 (2000)

FAPAS (2000) Report N 2301 (Series 23 Round 01) Central Science Laboratory MAFF London

Forthofer RN, Sul Lee E (1995) Introduction to bio-statistics: a guide to design, analysis and discovery. Academic Press, San Diego London

Freeling M, Bennet DC (1985) Ann Rev Genet 19: 297–323

Fuchs RL, Heeren RA, Gustafson ME, Rogan GJ, Bartnicki DE, Leimgruber RM, Finn RF, Hershman A, Berberich SA (1993) Biotechnology 11: 1537–1542

Gachet M, Martin GG, Vigneau F, Meyer G (1999) Trends Food Sci Technol 9: 380–388

Gadani F (2000) Personal communication

Gadani F, Bindler G, Pijnenburg H, Rossi L, Zuber J (2000) Beitraege zur Tabakforschung International 19(2): 85–96

Gierson E, Tucker GA, Keen J, Ray J, Bird CR, Schuch W (1986) Nucleic Acids Res 14: 8595-8603

Gilbert J, (1999) Food Control 10: 363–365

Gilliland G, Perrin S, Blanchard K, Bunn HF (1990) Proc Natl Acad Sci USA 87: 2725–2729

Golovkin MV, Abrahám M, Mórcocz S, Bottka S, Fehér A, Dudits D (1993) Plant Science 90: 41–52

González I, García T, Fernández A, Sanz B, Hernández PE, Marín R (1999) J Appl Microbiol 66: 231–236

Hardegger M, Brodmann P, Hermann A (1999) Eur Food Res Technol 209: 83–87

Hassan-Hauser C, Mayer W, Hörtner H (1998) Z Lebensm Unters Forsch A 206: 83–87

Hee Wan Kang, Yong Gu Cho, Ung Han Yoon, Moo Young Eun (1998) Plant Molecular Biology Reporter 16(1): 90

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Genome Res 6: 986–994

Heissenberger A (2000) Proceedings The European Commission's Joint Research Centre (JRC) and International Life Science Institute (ILSI) Joint Workshop on Method Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain. Brussels, Belgium 11–13 December 2000. In: J AOAC Int, *submitted*

Hellebrand M, Nagy M, Mörsel JT (1998) Z Lebensm Unters Forsch A 206: 237–242

Hemmer W (1997) Foods derived from genetically modified organisms and detection methods. BATS-Report 2/1997, Agency for Biosafety Research and Assessment of Technology Impacts of the Swiss Priority Programme Biotechnology of the Swiss National Science Foundation, Basel, Switzerland

Hergersberg M (1988) Molekulare Analyse des waxy Gens aus Solanum tubersorum und Expression von waxy antisense RNA in transgenen Kartoffen. PhD Thesis. University of Cologne, Germany

Higuchi R, Dollinger G, Walsh PS, Griffith R (1992) Bio/Technology 10: 413–417

Horwitz W (1995) Pure and Appl Chem 67: 331-343

Höhne M, Rosa C, Meyer R (2000) Proceedings The European Commission's Joint Research Centre (JRC) and International Life Science Institute (ILSI) Joint Workshop on Method Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain. Brussels, Belgium 11–13 December 2000 In: J AOAC Int, *submitted*

Howell M (2000) Proficiency Testing of Detection Methods for Genetically Modified Organisms: Proceeding of the Joint AOAC conference on Genetically Modified Organisms in the food chain, Munich

Hübner P (2000) Personal communication

Hübner P, Studer E, Häfliger D, Stadler M, Wolf C, Looser C (1999a) Accred Qual Assur 4: 292–298

Hübner P, Studer E, Lüthy J (1999b) Food Control 10: 353–358

Hübner P, Studer E, Lüthy J (1999c) Nature Biotechnology 17: 1137–1138

Hudspeth RL, Grula JW (1989) Plant Mol Biol 12: 579–589

Hupfer C, Hotzel H, Sachse K, Engel KH (1997) Z Lebensm Unters Forsch 205: 442–445

Hupfer C, Hotzel H, Sachse K, Engel KH (1998) Z Lebensm Unters Forsch A 206: 203–207

Hupfer C, Hotzel H, Sachse K, Moreano F, Engel KH (2000a) Eur Food Res Technol 212: 95–99

Hupfer C, Mayer J, Hotzel H, Sachse K, Engel KH (1999a) Eur Food Res Technol 209: 301–304

Hupfer C, Schmitz-Winnenthal J, Engel KH (1999b) Lebensmittelchemie 53: 2–20

Hurburgh CR, Rippke GR, Heithoff C, Roussel SA, Hardy CL (2000) Detection of genetically modified grains by near-infrared spectroscopy. Proceedings PITTCON 2000 – Science for the 21st Century, #1431. New Orleans, LA 12–17 March 2000 or Internet (2000) http://pittcon24857.omnibooksonline.com/

Hurst CD, Knight A, Bruce IJ (1999) Molecular Breeding 5: 579–586

Ibrahim SM, Lofts RS, Jahrling PB, Hencal EA, Weedn VW, Northrup MA, Belgrader P (1998) Anal Chem 70: 2013–2017

International Conference on Harmonisation (1996) Validation of Analytical Procedures: Methodology. http://www.ifpma.org/pdfifpms/q2b.pdf

ILSI Europe Report on the European Commission's Joint Research Centre (JRC) and International Life Science Institute (ILSI) Joint Workshop on Method Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain. Brussels, Belgium 11-13 December 2000

Ishii Y, Yanagida T (2000) Single Mol 1: 5–16

Jaccaud E, Höhne M, Meyer R (2000) Proceedings The European Commission's Joint Research Centre (JRC) and International Life Science Institute (ILSI) Joint Workshop on Method Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain. Brussels, Belgium 11–13 December 2000. In: J AOAC Int, *submitted*

Jackson PE, Scholl PF, Groopman JD (2000) Molecular Medicine Today 6: 271–276

Jankiewicz A, Broll H, Zagon J (1999) Eur Food Res Technol 209(2): 77–82

Johnson MR, Wang K, Smith JB, Heslin MJ, Diasio RB (2000) Anal Biochem 278: 175–184

Jongedijk E, de Schutter AAJM, Stolte T, van den Elzen PJM, Cornelissen BJC (1992) Bio/Technology 10: 422–429

Kai E, Sawata S, Ikebukuro K, Iida T, Honda T, Karube I (1999) Anal Chem 71: 796–800

Kay RA, Chan M, Daly M, McPherson J (1987) Science 236: 1299–1302

Khandurina J, McKnight TE, Jacobson SC, Waters LC, Foote RS, Ramsey JM (2000) Anal Chem 72: 2995–3000

Kirihara JA, Hunsberger JP, Mahoney WC, Messing JW (1988) Mol Gen Genet 211: 477–484

Köhler T, Laßner D, Rost AK, Thamm B, Pustowoit B, Remke H (1996) Quantitation of mRNA by DNA polymerase chain reaction – Non-radioactive PCR methods. Springer, Berlin Heidelberg New York

Kok EJ, Aarts Hjm, Van Hoef AMA, Kuiper HA (2000) DNA methods: Critical review of innovative approaches Proceedings The European Commission's Joint Research Centre (JRC) and International Life Science Institute (ILSI) Joint Workshop on Method Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain. Brussels, Belgium 11–13 December 2000. In: J AOAC Int, *submitted*

Köppel E, Stadler M, Lüthy J, Hübner P (1997) Mitt Geb Lebensm Hyg 88: 164–175

Köppel-Overkamp R (2000) Personal communication

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola SV (1993), Bio/Technology 11: 194–200

Koziel MG, Desai NM, Lewis KS, Kramer VC, Warren GW, Evola SV, Crossland LD, Wright MS, Merlin EJ, Launis KL, Rothstein SJ, Bowman CG, Dawson JL, Dunder EM, Pace GM, Suttie JL (1997) Synthetic DNA sequence having enhanced insecticidal activity in maize. US Patent No. 5,625,136, application No. 951715

Krabel D, Vornam B, Herzog S (1998) Angew Bot 72: 10–13

Krech A (1997) Mais "high mobility group' Proteine: Isolierung und Analyse von Genkonstrukten. PhD Thesis. Albert-Ludwigs Universität, Freiburg

Kriete G, Perlick AM, Pühler A, Broer I (1996) Induzierbare männliche Sterilität bei transgenen Tabakpflanzen. 3rd GPZ-Tagung, Transgenic Plants – From Lab to the Field. Köln, Germany 28–29 February 1996

Kuipers GJ, Vreem JTM, Meyer H, Jacobsen E, Feenstra WJ, Visser RGF (1992), Euphytica 59: 83–91

Laken SJ, Jackson PE, Kinzler KW, Vogelstein B, Strickland PT, Groopman JD, Friesen MD (1998) Nature Biotechnology 16:1352–1356

Landgraf A, Reckmann B, Pingoud A (1991) Anal Biochem 198(1): 86–91

Laurie DA, Bennett MD (1985) Heredity 55: 307–313

Lemieux B, Aharoni A, Schena M (1998) Molecular Breeding 4: 277–289

Leone G, van Schijndel H, van Gemen B, Kramer FR, Schoen CD (1998) Nucleic Acids Res 26: 2150–2155

Lipp M, Anklam E (2000) J AOAC Int 83(4): 919–927

Lipp M, Bluth A, Eyquem F, Kruse L, Schimmel H, van den Eede G, Anklam E (2001) Eur Food Res Technol 212: 497-504

Lipp M, Brodmann P, Pietsch K, Pauwels J, Anklam E (1999) J AOAC Int 82: 923–928

Lipton CR, Dautlick JX, Grothaus GD, Hunst PL, Magin KM, Mihaliak CA, Rubio FM, Stave JW (2000) Food and Agricultural Immunology 12: 153–164

LMBG L 23.01.22 (1998), Official Collection of Test Methods in accordance with Article 35 LMBG (Lebensmittel-und-Bedarfsgegenständegesetz), classification no. L 23.01.22: Detection of a genetic modification of soybeans by amplification of the modified DNA sequence by means of the polymerase chain reaction (PCR) and hybridisation of the PCR product with a DNA probe. German Federal Foodstuffs Act – Food Analysis, article 35, L 23.01.22-1. Beuth, Berlin Köln

LMBG L 24.01-1 (1997), Official Collection of Test Methods in accordance with Article 35 LMBG (Lebensmittel-und-Bedarfsgegenständegesetz), classification no L24.01-1: Detection of a genetic modification of potatoes by amplification of the modified DNA sequence using the polymerase chain reaction (PCR) and hybridisation of the PCR product with a DNA probe. German Federal Foodstuffs Act – Food Analysis, article 35, L 24.01-1. Beuth, Berlin Köln

LMBG L 25.03.01 (1999), Official Collection of Test Methods in accordance with Article 35 LMBG, (Lebensmittel-und-Bedarfsgegenständegesetz), classification no. L 25.03.01: Detection of a genetic modification of tomatoes by amplification of the modified DNA sequence using the polymerase chain reaction (PCR) and hybridisation of the PCR product with a DNA probe. German Federal Foodstuffs Act – Food Analysis, article 35, L 25.03.01. Beuth, Berlin Köln

Longstaff M., Edmonds HS, Newell CA. (1995) Plant Molecular Biology Reporter 13:363–368.

Lüthy J (1999) Food Control 10: 359–361

Matsuoka T, Kawashima Y, Akiyama H, Miura H, Goda Y, Kusakabe Y, Isshiki K, Toyoda M, Hino A (2000) J Food Hyg Soc Japan 41(2): 137–143

McKenzie MJ, Mett V, Jameson PE (2000) Plant Cell Rep 19: 286–289

McKillip J, Drake M (2000) J Food Prot 63(7): 855–859

Melzak KA, Sherwood CS, Turner RFB, Haynes CA (1996) J Colloid Interface Sci 181: 635–644

Messens K, Theuns I, Dewettinck K, Van Bockstaele E, Huyghebaert A, De Loose M (1999) Detection of genetically modified organisms in food. Med. Fac. Landbouww., Univ. Gent, 64/5b, Gent, Belgium

Meyer R (1995) Z Lebensm Unters Forsch 201: 583–586

Meyer R (1999) Food Control 10: 391–399

Meyer R, Chardonnens F, Hübner P, Lüthy J (1996) Z Lebensm Unters Forsch 203: 339–344

Meyer R, Jaccaud E (1997) Detection of genetically modified soya in processed food products: development and validation of PCR assay for the specific detection of glyphosate-tolerant soybeans. In: Amado R, Battaglia R (eds) Proceedings 9th European Conference on Food Chemistry. Authenticity and adulteration of food – The analytical approach, vol 1. Interlaken, Switzerland 24–26 September 1997, pp 23–28

Minunni M, Mascini M, Motti C, Dainese E, Di Matteo A, Chen B, Cozzani I (2000a) In: Italian Journal of Biochemistry, *in press*

Minunni M, Tombelli S, Chiti G, Mascini M (2000b) Biosensor approach for genetically modified organisms (GMO) detection. Euroanalysis XI, 11th Edition of the European Conference on Analytical Chemistry, Lisboa, Portugal 3–9 September 2000

Mishalani E (1999) Inside Lab Manag (Febr) pp 14-16

Moehrle M, Krech A, Winterhalter T, Zeltz P (2000) Proceedings The European Commission's Joint Research Centre (JRC) and International Life Science Institute (ILSI) Joint Workshop on Method

Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain. Brussels, Belgium 11–13 December 2000. In: J AOAC Int, *submitted*

Monsanto (1993) Soybean / HT/Glyphosate tolerant, APHIS petition No. 93-258-01p. Biotechnology Permits, USDA, APHIS, BBEP, Riverdale, MD 20737

Monsanto (1995) Corn / IR/Lepidopteran resistant. APHIS petition No. 95-093-01p. Biotechnology Permits, USDA, APHIS, BBEP, Riverdale, MD 20737

Muddiman DC, Anderson GA, Hofstadler SA, Smith RD (1997) Anal Chem 69(8): 1543–1549

Murray HG, Thompson WF (1980) Nucleic Acids Res 8: 4321–4325

Nasarabadi S, Milanovich F, Richards J, Belgrader P (1999) BioTechniques 27(6): 1116– 1118

Neff WE, Mounts TL, Rinsch WM, Konishi H, El-Agaimy MA (1994) J Am Oil Chem Soc 71: 1101–1109

Neff WE, Selke E, Mounts TL, Rinsch WM, Frankel EN, Zeitoun MAM (1992) J Am Oil Chem Soc 69: 111–118

Newton, CR and Graham A (1994) PCR BIOS Scientific Publishers Limited, Oxford

Padegimas L, Shullga OA, Skryabin KG (1993), Mol Biol 27: 583–585

Padgette SR, Kolacz KH, Delannay X, Re DB, LaVallee BJ, Tinius CN, Rhodes WK, Otero YI, Barry GF, Eichholz DA, Peschke VM, Nida LD, Taylor NB, Kishore GM (1995) Crop Science 35: 1451–1461

Pauli U, Liniger M, Zimmermann A (1998) Z Lebensm Unters Forsch 207: 264–267

Pauli U, Liniger M, Zimmermann A, Schrott M (2000) Mitt Lebensm Hyg 91: 491–501

Pauwels J, Kramer GN, Schimmel H, Anklam E, Lipp M, Brodmann P (1999a) The certification of reference materials of maize powder with different mass fractions of Bt-176 Maize EUR 18684 EN 15 pp. and IRMM soybeans CRM 410. GMOs with different mass fractions of Roundup-Ready® Soya. http://www.irmm.jrc.be/rm/irmm-crm-410.pdf

Pauwels J, Kramer GN, Schimmel H, Anklam E, Lipp M, Brodmann P (1999b) The certification of reference materials of maize powder with different mass fractions of Roundup-Ready® soya EUR 18683 EN 17pp. and IRMM maize CRM 411. Dried maize powder containing genetically modified Bt-176 maize. http://www.irmm.jrc.be/rm/irmm-crm-411.pdf

Pietsch K, Waiblinger HU, Brodmann P, Wurz A (1997) Dtsch Lebensm Rundsch 93(2): 35–38

Pijnenburg H (1998) Relative quantification calculation methods. First Swiss Sequence Detection Systems User Meeting. Wengen, Switzerland 3–4 December 1998

Plant Genetic Systems (1995) Corn / AP/HT/Male sterile/Phosphinotricin tolerant. APHIS petition No. 95-228-01p. Biotechnology Permits, USDA, APHIS, BBEP, Riverdale, MD 20737

Preston LR, Harker N, Holton T, Morell MK (1999) Plant Varieties and Seeds 12: 191–205

Raeymaekers L (1993) Anal Biochem 214: 582–585

Rasmussen RP (1994) The Rapid Cyclist 2(1): 1–5

Rich RL, Myszka DG (2000) Current Opinion in Biotechnology 11: 54–61

Ririe KM, Rasmussen RP, Wittwer CT (1997) Anal Biochem 254: 154

Rocha-Sosa M, Sonnewald U, Frommer W, Stratmann M, Schell J, Willmitzer L (1989) EMBO J 8: 23–29

Rochester DE, Winer JA, Shah DM (1986) EMBO J 5: 451–458

Rogan GJ, Dudin YA, Lee TC, Magin KM, Astwood JD, Bhatka NS, Leach JN, Sanders PR, Fuchs RL (1999) Food Control 10: 407–414

Rogan GJ, Ream JE, Berberich SA, Fuchs RL (1992) J Agric Food Chem 40: 1453–1458

Rogers SO, Bendich AO (1988) Plant Molecular Biology Manual A6: 1–10

Ross PL, Davis PA, Belgrader P (1998) Anal Chem 70: 2067–2073

Rossi L, Bindler G, Pijnenburg H, Isaac P, Giraud-Henry I, Mahe M, Orvain C, Gadani F (2000) Potential of molecular marker analysis for variety identification in processed tobacco (Nicotiana tabacum L.), *submitted*

Ryman N (1999) Statistical assessment of the proportion of GM grains in bulk consignment. EU-Project No. B4-3040/98/000272/MAR/E2. Final Report, 16 June 1999

Sakamoto K, Kusaba M, Nishio T (2000) Plant Cell Rep 19(4): 400–406

Sanders GWH, Manz A (2000) Trends in Analytical Chemistry 19: 364–378

Sanders PR, Winter JA, Barnason AR, Rogers SG, Fraley RT (1997) Nucleic Acids Res 15(4): 1543–1558

Schofield T (2000). Assay Validation in “Encyclopedia of Biopharmaceutical Statistics” ed. S-C Chow Marcel Dekker Inc, New York

Schweizerisches Lebensmittelbuch (1998) Molekularbiologische Methoden. In: Bundesamt für Gesundheit (ed) Eidgenössische Drucksachen und Materialzentrale, Bern, Switzerland chap 52B

Schwers S, Ehlich A, Kobsch S, Missel A, Loffert D (2000) QIAGEN News 3: 13–16

Sheehy RE, Kramer M, Hiatt WR (1988), Proc Natl Acad Sci USA 85: 8805–8809

Siler D, Minnick N, Dvorak N, Moore L, Isaac P, Crystele C, Malandro MS (2000) Quantitative detection of transgenes in genetically modified plants by Real-time PCR. Plant & Animal Genome VIII conference. San Diego, CA 9–12 January 2000

Singh M, Bandana, Ahuja PS (1999) Plant Molecular Biology Reporter 17(2): 171–178

Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, Schuch W, Grierson D (1988), Nature 334: 724–726

Smith CJS, Watson CF, Ray J, Bird CR, Morris PC, Schuch W, Gierson D (1990), Mol Gen Genet 224: 477–481

Smyth WF (1999) Trends in Analytical Chemistry 18: 335–346

Souteyrand E (1999) Analusis 27(7): 639–646

Spoth B, Strauss E (1999) Promega Notes Magazine 73: 23–25

Stave J (1999) Food Control 10: 367–374

Stave JW, Magin KM, Schimmel H, Lawruk T, Wehling P, Bridges AR (2000) Cereal Foods World 45: 497–501

Steinkeliner H, Korschhineck I (1998) Detection of recombinant viral coat protein in transgenic plants. In: Cunningham C, Porter AJR (eds) Methods in biotechnology. Recombinant proteins from plants, vol. 3. Humana Press, Totowa, New Jersey, pp 65–75

Studer E, Dahinden I, Lüthy J, Hübner P (1997) Mitt Geb Lebensm Hyg 88: 515–525

Studer E, Rhyner C, Lüthy J, Hübner P (1998) Z Lebensm Unters Forsch 207: 207–213

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Plant Mol Biol 17: 1105–1109

Taoufik Y, Froger D, Benoliel S, Wallon C, Dussaix E, Delfraissy JF, Lantz O (1998) Eur Cytokine Netw 9: 197–204

Taranenko NI, Allman SL, Golovlev VV, Taranenko NV, Isola NR, Chen CH (1998) Nucleic Acids Res 26(10): 2488–2490

Taton TA, Mirkin C, Letsinger RL (2000) Science 289: 1757–1760

Tengel C, Schüßler P, Sprenger-Haußels M, Setzke E, Balles J (2000) Dtsch Lebensm Rundsch 96: 129–135

Terry C, Parkes H (2000) Proceedings The European Commission's Joint Research Centre (JRC) and International Life Science Institute (ILSI) Joint Workshop on Method Development in Relation to Regulatory Requirements for the Detection of GMOs in the Food Chain. Brussels, Belgium 11–13 December 2000. In: J AOAC Int, *submitted*

Tyagi S, Bratu DP, Kramer FR (1998) Nature Biotechnology 16: 49–53

Tyagi S, Kramer FR (1996) Nature Biotechnology 14: 303–308

United States Department of Agriculture (1995) Grain Inspection Handbook – Book I –Grain Sampling. United States Department of Agriculture (USDA), Grain Inspection Packers and Stockyards Administration (GIPSA), Federal Grain Inspection Service, Washington DC or Internet http://www.usda.gov/gipsa/reference-library/handbooks/grain-insp/grbook1/gihbk1.htm

Vaïtilingom M, Pijnenburg H, Gendre F, Brignon P (1999) J Agric Food Chem 47: 5261–5266

Van den Eede G (2000) Personal communication

Van den Eede G, Lipp M, Eyquem F, Anklam E (2000) Validation of a double competitive polymerase chain reaction method for the quantification of GMOs in raw materials. EUR 19676 EN, European Commission Joint Research Centre, Institute for Health and Consumer Protection, Food Products and Consumer Goods Unit, Ispra, Italy

Vodkin LO, Rhodes PR, Goldberg RB (1983) Cell 34: 1023–1031

Von Ahsen N, Oellerich M, Schütz E (2000) Clin Chem 46: 156–161

Von Holst C (2000) Personal communication

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M (1995) Nucleic Acids Res 23(21): 4407–4414

Vroh Bi I, Harvengt L, Chandelier A, Mergeai G, Du Jardin P (1996) Plant Breeding 115: 205–206

Wang H, Qi M, Cutler A (1993) Nucleic Acids Res 21(17): 4153–4154

Waters LC, Jacobson SC, Kroutchinina N, Khandurina J, Foote RS, Ramsey JM (1998) Anal Chem 70: 5172–5176

Whitaker TB, Springer J, Defize P, de Koe J, Coker R (1995) J Assoc Off Anal Chem 78: 1010–1018

Whitecomb D, Theaker J, Guy SP, Brown T, Little S (1999) Nature Biotechnology 17: 804–807

Wittor H, Emig M, Betzl G, Hehlmann R, Leying H, Hochhaus A (1998) Detection and quantification of BCR-ABL transcripts using a novel Real-time approach with specific fluorescent hybridization probes. The American Society of Hematology 40th Annual Meeting. Miami, Florida 4–8 December 1998

Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP (1997) BioTechniques 22: 130–131, 134–138

Wittwer CT, Ririe KM, Rasmussen RP (1998) Fluorescence monitoring of rapid cycle PCR for quantification. In: Ferré F (ed) Gene quantification. Advanced biomedical technologies. Birkhäuser, Boston, pp 129–144

Wohlleben W, Arnold W, Broer I, Hillemann D, Strauch E, Pühler A (1988) Gene 70: 25–37

Wolf C, Scherzinger M, Wurz A, Pauli U, Hübner P, Lüthy J (2000) Eur Food Res Technol 210: 367–372

Wood DC, Vu LV, Kimack NM, Glennon JR, Ream JE, Nickson TE (1995) J Agric Food Chem 43: 1105–1109

Woolley AT, Hadley D, Landre P, deMello AJ, Mathies RA, Northrup MA (1996) Anal Chem 68: 4081–4086

Wurz A, Bluth A, Zeltz P, Pfeifer P, Willmund R (1999) Food Control 10: 385–389

Wurz A, Rüggeberg H, Brodmann P, Waiblinger HU, Pietsch K (1998) Dtsch Lebensm Rundsch 94: 159–161

Wurz A, Willmund R (1997) Identification of transgenic glyphosate-resistant soybeans. In: Schreiber GA, Bögl KW (eds) Foods produced by means of genetic engineering. 2nd Status Report. BgVV-Heft 1/1997, Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany, pp 115–117

Yang JS, Yu TA, Cheng YH, Yeh SD (1996) Plant Cell Rep 15: 459–464

Zeneca (1995) Potato / IR/Colorado potato beetle resistant. APHIS petition No. 95-290-01p. Biotechnology Permits, USDA, APHIS, BBEP, Riverdale, MD 20737

Zimmermann A, Lüthy J, Pauli U (1998a) Z Lebensm Unters Forsch 207: 81–90

Zimmermann A, Hemmer W, Liniger M, Lüthy J, Pauli U (1998b) Lebensm -Wiss Technol 31: 664–667