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Food and Chemical Toxicology 42 (2004) 1157–1180

Food and Chemical Toxicology

www.elsevier.com/locate/foodchemtox

Detection and traceability of genetically modified organisms in the food production chain

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Received 16 November 2003; accepted 4 February 2004

Abstract

Both labelling and traceability of genetically modified organisms are current issues that are considered in trade and regulation. Currently, labelling of genetically modified foods containing detectable transgenic material is required by EU legislation. A proposed package of legislation would extend this labelling to foods without any traces of transgenics. These new legislations would also impose labelling and a traceability system based on documentation throughout the food and feed manufacture system. The regulatory issues of risk analysis and labelling are currently harmonised by Codex Alimentarius. The implementation and maintenance of the regulations necessitates sampling protocols and analytical methodologies that allow for accurate determination of the content of genetically modified organisms within a food and feed sample. Current methodologies for the analysis of genetically modified organisms are focused on either one of two targets, the transgenic DNA inserted- or the novel protein(s) expressed- in a genetically modified product. For most DNA-based detection methods, the polymerase chain reaction is employed. Items that need consideration in the use of DNA-based detection methods include the specificity, sensitivity, matrix effects, internal reference DNA, availability of external reference materials, hemizygosity versus homozygosity, extrachromosomal DNA, and international harmonisation. For most protein-based methods, enzyme-linked immunosorbent assays with antibodies binding the novel protein are employed. Consideration should be given to the selection of the antigen bound by the antibody, accuracy, validation, and matrix effects. Currently, validation of detection methods for analysis of genetically modified organisms is taking place. In addition, new methodologies are developed, including the use of microarrays, mass spectrometry, and surface plasmon resonance. Challenges for GMO detection include the detection of transgenic material in materials with varying chromosome numbers. The existing and proposed regulatory EU requirements for traceability of genetically modified products fit within a broader tendency towards

Abbreviations: CaMV, cauliflower mosaic virus; CEN, Comité Européen de Normalisation; Cry1Ab, an insecticidal crystal protein from *Bacillus thuringiensis*; DNA, deoxyribonucleic acid; *E. coli*, *Escherichia coli*; ELISA, enzyme-linked immunosorbent assay; EN, European standard; ENGL, European Network of GMO Laboratories; ENTRANSFOOD, European network safety assessment of genetically modified food crops; EPSPS, enolpyruvylshikimate phosphate synthase; EU, European Union; F&F, proposal of the European Commission for a regulation on GM food and feed; FAO, Food and Agriculture Organisation of the United Nations; FRET, Fluorescence Resonance Energy Transfer; GM, genetically modified; GMO, genetically modified organism; HACCP, hazard analysis and critical control points; IP, identity preservation; ISO, International Organisation for Standardisation; ISTA, International Seed Testing Association; JRC, Joint Research Centre; KELDA, Kernel Lot Distribution Assessment Project; LOD, limit of detection; LOQ, limit of quantitation; MALDI, matrix-assisted laser desorption/ionisation; MS, mass spectroscopy; OECD, Organisation for Economic Cooperation and Development; PCR, polymerase chain reaction; QPCRGMOFOOD, EU project on reliable, standardised, specific, quantitative detection of genetically modified foods; RNA, ribonucleic acid; SNP, single nucleotide polymorphism; SPR, surface plasmon resonance; T&L, proposal of the European Commission for a regulation on traceability on labelling of GMOs and products obtained from GMOs; TC, technical committee; TILLING, technique for targeting induced local lesions in genomes; TOF, time of flight; UK, United Kingdom; US, United States of America; USDA/GIPSA, Grain Inspection, Packers, and Stockyards Administration of the US Department of Agriculture; UV, ultraviolet; WHO, World Health Organisation of the United Nations.

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0278-6915/$ - see front matter
doi:10.1016/j.fct.2004.02.018

traceability of foods in general and, commercially, towards products that can be distinguished from each other. Traceability systems document the history of a product and may serve the purpose of both marketing and health protection. In this framework, segregation and identity preservation systems allow for the separation of genetically modified and non-modified products from "farm to fork". Implementation of these systems comes with specific technical requirements for each particular step of the food processing chain. In addition, the feasibility of traceability systems depends on a number of factors, including unique identifiers for each genetically modified product, detection methods, permissible levels of contamination, and financial costs. In conclusion, progress has been achieved in the field of sampling, detection, and traceability of genetically modified products, while some issues remain to be solved. For success, much will depend on the threshold level for adventitious contamination set by legislation.

Keywords: Traceability; Detection methods; Sampling; Regulation

1. Introduction

Issues related to detection and traceability of genetically modified organisms (GMOs) are gaining world wide interest due to the ever-increasing global diffusion and the related socio-economical implications. The interest of the scientific community into traceability aspects has increased simultaneously.

Progress, both in sampling and detection methodologies and in traceability strategies, strongly influences the potential for adequate implementation and maintenance of legislation and labelling requirements. Crucial factors in this respect are the numbers of the GMOs involved and international agreement on traceability. The availability of reliable traceability strategies may increase public trust in transparency in GMO issues.

The detection and identification of GMOs represents a relatively new area of diagnostics in which much progress has already been achieved with DNA- and protein-based methods. In this paper, the section dealing with the detection highlights the current state of the art of the methodology. In addition, it also indicates current needs and limitations that hamper further developments in the area of GMO detection. Obvious needs are (1) information on GMOs developed worldwide, (2) availability of material for stated purposes, and (3) transparency of information.

The paper also deals with new diagnostic methodologies, such as the microarray-based methods that allow for the simultaneous identification of the increasing number of GMOs on the global market in a single sample (some of these techniques have also been discussed for the detection of unintended effects of genetic modification by Cellini et al., 2004). Emphasis is also given to the quality assurance of the analytical data represented by the adequate availability of suitable Certified Reference Materials for different applications and the need for harmonised guidelines for validation studies.

The implementation of adequate traceability systems requires more than technical tools alone and is strictly linked to labelling constraints. The more stringent the labelling requirements, the more expensive and difficult the associated traceability strategies are to meet these requirements.

This paper summarises the outcome of the discussions of Working Group IV (Detection and Traceability of GMOs) of the ENTRANSFOOD Thematic Network on the Safety Assessment of Genetically Modified Food Crops.

2. Regulation

2.1. Current regulation

The current EU legislation for genetically modified organisms (GMOs) regulates issues concerning environmental aspects and food and feed safety, procedures for commercialisation and labelling provisions. In the environmental aspects the contained use and the deliberate release of GMOs into the environment are included. Furthermore, an important legislative package, which will further complete and reinforce the current legislation, has been presented by the Commission but not approved yet at present.

The milestones of the European Union's GMO legislation are represented by Directive 90/220 on the introduction of GMOs into the environment and Regulation 258/97 on novel foods and novel food ingredients (European Commission, 1990, 1997a). Directive 90/220 was recently replaced by Directive 2001/18, which, among others, introduces the concept of traceability (European Commission, 2001a). Directive 90/220, which first provided the current definition of GMOs, put in place a safety-based step-by-step approach and a case-by-case assessment of the risks to human health and the environment. It was mandated that the whole assessment procedure be completed prior to the environmental release and, before Regulation 258/97 came into force, the introduction on the market of food products containing or consisting of GMOs. Under Directive 90/220, a total of 18 authorisations have been granted for the placing on the market of GMOs, among which two cover the use in food (one maize and one soybean variety) and eight cover the use in feed (one for

soybean, four for maize, and three for rape). At present, Regulation 258/97 and Directive 2001/18 have taken over Directive 90/220. A detailed safety assessment approach for GM crop-derived foods based on most recent developments in the area is described in the paper by König et al. (2004).

Regulation 258/97 on Novel Foods and Food Ingredients, commonly referred to as the "Novel Foods Regulation", entered into force in 1997. Currently, it still regulates the marketing and labelling of a broad category of "novel" foods including those produced by new technologies (such as GMOs) and foods that do not have a history of safe use in Europe. The core of the Regulation is represented by the following principles: (1) novel foods (and derived ingredients) must be safe and not be nutritionally disadvantageous for the consumer, and (2) the consumer has the right to be informed whenever a novel food or food ingredient is no longer equivalent to an existing food or food ingredient. The Regulation also covers the procedure for placing the novel foods on the market. This procedure strongly involves both the Member States and the Commission, and may follow either one of two lines, authorisation or notification, the latter being allowed in the case of substantial equivalence of the product to its conventional counterpart that is already on the market. No genetically modified (GM) food has so far been authorised under the Novel Foods Regulation, but thirteen products, which were substantially equivalent to already existing conventional foods, have been notified. An actual list of GMO products approved or pending approval under Directives 90/220 and 2001/18 and Regulation 258/97, together with other relevant information on European legislation can be found on the Internet (European Commission, 2003; Belgian Biosafety Server, 2003).

It is further stipulated that in order to provide the consumer with the proper information, GMOs, as well as other novel food products, must be labelled if they differ from their conventional counterparts. Product choice as one of the factors that determine public acceptance of GM foods is described in the paper by Frewer et al. (2004). Initially, no special labelling was stipulated for Bt-176 maize and Roundup Ready soybeans, because they had been approved in the EU through Commission Decisions 96/281 (soybean; European Commission, 1996) and 97/98 (maize; European Commission, 1997b) before the Novel Foods Regulation came into force. Since thousands of different foodstuffs contained soybean and maize products and since it was agreed that consumers had the right to be informed about genetically engineered ingredients, the Commission has amended the labelling requirements. Regulation 1139/98 stated that products from Bt-176 maize and Roundup Ready soybean had to be labelled if the new protein or genetically modified DNA were detectable in the end product intended for consumption (European Commission, 1998a). In this case, the label "produced from genetically modified soybeans" or "produced from genetically modified maize" was mandatory. This meant that food manufacturers needed not to label a final product when a genetically engineered raw material had been technically treated in such a way that neither the new DNA nor the protein could be detected. For example, this applies to hydrolysed soy protein or to refined oil. Regulation 1139/98 was further amended by introducing a tolerance level for any adventitious and unintended contamination with GMO-derived materials. According to Regulation 49/2000, labelling in this situation is not required when the percentage of GM material is not higher than 1% of the food ingredient. For values less than the threshold level, specific labelling is not compulsory, provided the producer can demonstrate that the presence of GM material is adventitious (European Commission, 2000a). Finally, Regulation 50/2000 concerns additives and flavourings that have been genetically modified or have been produced from genetically modified organisms and provides for labelling in the case of presence in the finished product of additives and flavourings derived from genetic modification without any threshold level (European Commission, 2000b). For non-authorised GMOs, no tolerance level has been established, the threshold value therefore being the detection limit of the employed methods.

2.2. New proposals

In response to Action 6 of the White Paper on Food Safety (European Commission, 2000c), the European Commission adopted two new proposals on 25 July 2001 to: (1) revise the authorisation system for GMO-derived food and feed, (2) reinforce the current labelling provisions, (3) introduce the labelling of feed, and (4) define an EU system for the traceability of GMOs. The package of proposals, to be adopted on the basis of a co-decision procedure between the Council of Ministers and the European Parliament, consists of (1) a proposal on GM food and feed, including bulk commodities and processed foods (F&F; European Commission, 2001b), which was recently amended (European Council, 2003a), and (2) a proposal (European Commission, 2001c) for traceability and labelling of GMOs and products obtained by GMOs (T&L), which was also recently amended (European Council, 2003b). It was emphasised that the achievement of a high level of environment and health protection and an increased public confidence were the most relevant overall objectives of the proposals.

In the F&F proposal, innovative procedures for authorisation include the scientific risk assessment, both for the environment and for human and animal health,

to be undertaken by the European Food Safety Authority. Both scientific risk assessment and authorisation of GMOs and GMO-derived food and feed products should be based on the "one door-one key" basis and the authorisation should be granted for a 10 years period. The proposed regulation extends the current labelling provisions to all food and feed items derived from GMOs, including those in which GMO-derived DNA or protein is no longer detectable with current methodologies (e.g., in the case of refined oils). Provisions for the safety assessment and authorisation procedure in Regulation 258/97 remain in place for non-GMO-derived novel foods.

The traceability and labelling (T&L) proposal is considered interrelated and complementary to the F&F proposal. The main objectives of the proposed T&L regulation are: (1) to facilitate "the targeted monitoring of potential effects on human health or the environment", (2) to withdraw products in the case of an unforeseen risk to human health or the environment, and (3) to facilitate the control and the implementation of labelling requirements. According to the proposed regulation, all GMO-derived products (including seed, bulk commodities used for processing, food items, and animal feed) that contain GM material must be labelled as such when sold in the EU. Since there are technical difficulties in segregating crops, the proposed regulation allows the adventitious presence of up to 0.9% of GMO-derived materials for EU-approved events in food and feed items. In this case, operators must ensure that every effort was made to exclude those events from the products. For other EU-unapproved varieties, the proposal maintains zero tolerance, unless they have already received a positive advice for marketing. In the latter case, a threshold level of 0.5% may be applied. Meat produced from animals fed with biotech feed does not require specific labelling.

The T&L proposal provides the definition of traceability for GMOs and methods for its implementation. In the case of products containing or consisting of GMOs, the information to be provided by the operator includes whether the product contains or consists of GMOs and the unique code(s) related to the GMO(s) contained in the product (the unique code must identify the authorised transformation event). For items produced from GMOs, information on the event from which the item is produced is not mandatory. The provisions for the unique code imply the development of an appropriate harmonised system. Current work that establishes such unique codes for GM varieties is ongoing under the umbrella of OECD.

Labelling must be present at the time when the items are first placed on the European market. Moreover, all GMO-related information must be transmitted to all future purchasers within the European supply chain and be retained for a 5 years period.

The European Network of GMO Laboratories (ENGL) under co-ordination of the European Joint Research Centre in Ispra/Italy, may be part of the new EU legislation on GMO food and feed that is now being formulated. The ENGL shall assist the JRC as the EU reference laboratory with validation and evaluation of detection methods, in particular through setting up interlaboratory collaborative trials.

2.3. International harmonisation

The Codex Alimentarius Commission has recently taken the leadership in the negotiations on definition of standards and guidelines in the GMOs issue in general and on international systems of product tracing. The Codex ad hoc Task Force on Food Derived from Modern Biotechnology recently issued its principles of the risk analysis of food derived from biotechnology, as well as guidelines for the safety assessment of foods derived from genetically modified plants and micro-organisms (FAO/WHO, 2003a). In addition, the Codex Committee on Food Labelling is currently developing guidelines for labelling of GM foods (FAO/WHO, 2001). In a more general sense, the Codex Committee on Food Import and Export Certification and Inspection Systems, the Committee on Food Labelling, and Committee on Food Hygiene are both working on different aspects related to the issue of traceability (FAO/WHO, 2003b).

3. Sampling

The evaluation of the GM materials present in a lot entails relevant implications for the trade and food production in view of the differences in legislation and in GMO acceptance worldwide. Determination of the content of GMOs in raw materials is subjected to errors during the various stages of the "diagnostic chain" (sampling, sub-sampling, and analysis).

Since, in most cases, GMOs are non-homogeneously distributed in the bulk, the variance associated with the sampling step is likely to represent the major contribution to the overall variance. The lower the GMO concentration is, the more relevant the effect of different sampling strategies will be. The experience with sampling methodologies for the analysis of mycotoxins provides the basis for provisional sampling schemes that can be applied to GMOs and in many European countries. Directive 98/53 on sampling and analysis of certain contaminants in foodstuffs was the sampling plan first suggested and still used as applicable to GMOs (European Commission, 1998b).

In general, sampling strategies have to take a wide variety of parameters into account, for example the nature of the analyte/foodstuff and distribution of the analyte

in the bulk. Quite a few organisations have already approached the problem of sampling for GMO-derived materials. In the process of defining a sampling plan for bulk products, the main parameters that should be statistically taken into account include lot size and uniformity, accepted risks (tolerances), and adopted testing methods, while parameters to be settled include increment size, rate of increment sampling, and preparation of the sample prior to the analysis (Kay and Paoletti, 2002). Pragmatic aspects, for example used/available sampling facilities (collection of flowing grains during loading/unloading or sampling in an immobile bulk) and costs.

Existing sampling plans, summarised in Table 1, are heterogeneous in many aspects. Many of them have not been specifically developed for GMOs. Some sampling plans, such as CEN (not yet finalised; Comité Européen de Normalisation) and USDA/GIPSA (2000, 2001) are aimed at GMO seeds or grains specifically. Brera et al. (personal communication) developed a sampling plan based on the development of a laboratory-scale model to be transferred to the real situation for the implementation of a whole procedure addressed to reduce the total variance associated with the GMO determination in a lot from the sampling to the analysis step. Kay and Paoletti (2002) published an overview of the sampling strategies for the screening of large grain shipments, primary ingredients, and of specific (GM) ingredients in final food products. The different plans were compared with each other with respect to lot size, sampling rates, increments, and preparation of the laboratory samples, while many differences subsisted for these parameters. It was observed that only a few plans were based on a sound statistical approach (Kay and Paoletti, 2002).

One of the priorities of the recently installed ENGL is to identify and develop appropriate sampling strategies to support EU legislation (ENGL, 2003; EC-JRC, 2003a). In this respect, considerable efforts are currently made by the KELDA project (KELDA, 2003). The requested level of certainty of the sampling plan will be a decisive factor in the selection process: it may be desirable to keep the level of uncertainty as low as possible in the case of seeds, while for foodstuffs, it is strictly dependent on the threshold level for the adventitious contamination. In general, acceptable sampling errors are directly associated with the evaluation of both the risk for the consumer, defined as the acceptance of lots above predetermined limits, and the risk for the producer, defined as the rejection of lots below legal limits. Therefore, the adoption of reliable sampling procedures and the definition of the error related to the sampling methodologies are very relevant for all parties involved. A quantitative evaluation of the errors associated with specific sampling plans for GMOs has not yet been performed. It should be kept in mind that such

Table 1
Comparison of sampling approaches for grains lots (adapted from Kay, 2002)

Source	Bulk size(s)	Tolerance	Bulk sample	Laboratory sample	Increments	Increments size
ISTA (1)	Varies according to species: 10,000 kg to 40,000 kg (max)	5%	1 kg	1 kg (approx. 3,000 maize kernels) (for analysis of contamination by other seed varieties)	One increment per 300 kg to 700 kg	Not indicated
USDA/GIPSA	Up to 10,000 bushels (approx. 254,000 kg), or 10,000 sacks if the lot is not loose	5%	Equivalent to laboratory sample	approx. 2.5 kg, but not less than 2 kg	3 cups or 1 cut per 500 bushels (approx. 12,000 kg)	1.25kg
USDA/GIPSA, StarLink™ (2)	Follows general USDA/GIPSA guidelines		Minimum 3 times laboratory sample, in practice approx. 2.5 kg	2400 kernels		
ISO 13690 (3)	Up to 500,000 kg	Not indicated	Not indicated	>1 kg (for kernels)	15 to 33 (static <50,000 kg), "as many as possible" free-flowing	Stated as 0.2 kg to 0.5 kg, but in practice up to 5 kg
ISO 542 (4)	Up to 500,000 kg	Not indicated	100 kg	2.5 kg to 5 kg	15 to 33 for loose bulk (up to 500,000 kg)	Not specified
EU Dir.98/53 (5)	No limit if not separable, otherwise up to 500,000 kg	20%	30 kg (lot size 50,000 kg) 1–10 kg (lot size <50,000 kg)	10 kg	Up to 100	0.3 kg
CEN (6)	Up to 500,000 kg	Not indicated	20 times laboratory sample (i.e. 60 kg)	100,000 kernels	As for ISO 13690, except the number of depths per sample point not specified	Stated as 0.5 kg, but in practice up to 5 kg, if ISO 542 fully applied
FAO/WHO (7)	Discussed, but not specified	Not indicated	Various proposals	Not discussed	Not indicated	Not indicated

(1) ISTA, 2003; Bould, 1986; (2) USDA/GIPSA, 2000; USDA/GIPSA, 2001; (3) ISO, 1999; (4) ISO, 1990; (5) European Commission, 1998b; (6) CEN, 2001; (7) FAO/WHO, 2002.

errors must be matched well with the current and proposed threshold values. In addition, it is necessary to verify the feasibility of the theoretical sampling procedures from the pragmatic point of view.

In conclusion, many sampling plans are currently available for bulk products with only some of them specifically addressed to GMOs. Relevant work is ongoing at ENGL level in order to match the theoretical approach with the real situation and reach a compromise between pragmatic and statistical approaches. Studies should be statistically based in order to be able to evaluate the compatibility between the set threshold and uncertainties/variance of the sampling plans.

4. GMO detection with DNA-based analytical methods

A GMO is usually defined as a living organism whose genetic composition has been altered by means of gene technology. This involves DNA isolation, defined DNA modification, and transfer of DNA into the genome of the target organism that successively becomes a GMO. This process is referred to as the transformation event. Normally, new gene functions are inserted into the GMO, but new techniques have been developed that make targeted knock-out of existing genes possible, among others in higher organisms, such as food plants (Terada et al., 2002). A typical insert (gene construct) in a GMO is composed of at least three elements: (1) the promoter element, which functions as an on/off switch for reading of the inserted/altered gene; (2) the gene that has been inserted/altered, which is coding for a specific selected feature; (3) the terminator element, which functions as a stop signal for reading of the inserted/altered gene. In addition, several other elements can be present in a gene construct and their function is usually to control and stabilise the function of the gene, demonstrate the presence of the construct in the GMO, or facilitate combination of the various elements of the construct. A gene construct must be integrated in the genome (the natural genetic background) of the organism to become stably inherited.

The integration process itself is complex and largely beyond human control and in addition to one or more copies of the construct, fragments of the constructs may become inserted and stably integrated into the recipient genome, depending on the transformation strategy (Windels et al., 2001).

Other breeding techniques than gene technology are used to modify DNA and increase genetic variability, such as chemical and irradiation mutagenesis. Although these techniques also involve genetic modifications, they do not involve the set of techniques defined as gene technology. Therefore, in most countries (not Canada and US), such techniques were defined to be outside the regulatory framework for authorisation and labelling for GMOs emerging in the 1990s. However, there are several scientific issues in common for GMOs and varieties established by other breeding techniques. Common for all these technologies is that the genetic material is modified with the objective of creating new and more useful varieties. A major difference is the degree of targeting. Gene technology is considered a highly targeted technology, which may involve few to moderate numbers of changes and mainly tend to seek modification at a single locus, whereas alternative mutagenic technologies usually create a large number of more or less random changes. On the other hand, a unique feature of gene technology is that it allows for transfer of genetic material across species barriers. The degree of targetedness is of central relevance for the detectability, since better knowledge of the genetic changes means that it is easy to develop methods to detect the genetic modifications. The recently developed TILLING technique (McCallum et al., 2000) is a modification and improvement of mutagenesis breeding, involving screening of the mutations. Thus, it improves the knowledge of the genetic changes and also the potential for their detection, and reduces the detectability gap between gene technology and mutagenesis breeding.

4.1. DNA-based analytical methods

Since GMOs are the result of genetic modifications, the most direct detection methods are those that target the genetic modification itself, i.e. the modified DNA. Other methods are available for some GMOs, in particular protein-based methods targeting the product resulting from the genetic modification. These methods, however, are unable to detect a genetic modification if the modified gene is inactive in the cells from which an analytical sample is derived, and they can not be used to distinguish between GMOs modified to produce the same protein, e.g. authorised and unauthorised.

At present, the most commonly used DNA-based methods involve amplification of a specific DNA with the PCR technique. The most basic technique for demonstration of the presence of amplified and/or hybridised DNA or RNA sequences is gel electrophoresis, a technique that allows the quantity and size of the DNA to be estimated. The identity of the amplified DNA may be further verified by DNA sequencing or digestion of the DNA with restriction enzymes followed by fragment analysis to determine the size of the resulting fragments. Several reviews of available DNA-based detection methods have been published recently (e.g. Anklam et al., 2002; Holst-Jensen et al., 2003).

Gel electrophoresis is normally used for a qualitative detection of PCR-amplified DNA. The first PCR-based quantitative detection methods developed also involved gel electrophoresis and a competitive quantification strategy. Competitive co-amplification of both an artificially

constructed DNA that is added in known quantities and the target in the sample (double competitive PCR) ideally maintains the initial ratio of the targets throughout the reaction, while the quantitative relationship is determined by image analysis of dilution series of the DNA (Gilliland et al., 1990). One problem with double competitive PCR is that it involves extensive handling and pipetting of amplified DNA, posing a significant risk of carry-over contamination. Furthermore, it is difficult to convert this type of assay into a standard procedure.

At present, real-time PCR is the most commonly used technology for quantification of GMOs. The amount of product synthesised during the PCR is measured in real-time by detection of fluorescence signal produced as a result of specific amplification. Real-time PCR requires special thermal cycles and usually the addition of specific fluorescent probes (unspecific fluorescence can also be detected using DNA binding fluorescent dyes, e.g. SYBR Green). Quantification takes place in the logarithmic phase of the PCR reaction (log-linear phase). Several types of fluorescent probes are available that will emit fluorescent light corresponding to the amount of synthesised DNA. The use of target specific probes, i.e. probes with binding affinity only to a very specific DNA sequence, evidently adds one more level of specificity to the test. An advantage of real-time PCR is the fact that vials with the PCR product do not have to be reopened to proceed with the verification step and thus the risk of carry-over contamination in routine analysis is reduced.

As for double competitive PCR, the quantitative estimate is established by comparing the relative ratios of a GMO-specific target sequence and a reference target sequence, usually a species-specific sequence. There is normally a linear correlation between the quantity of GMO and the quantity of genetically modified DNA. This correlation is less obvious between the quantity of GMO and protein/RNA.

4.2. Available methods of GMO detection

On a world wide basis, there are presently more than 100 events authorised by a competent authority for food and/or feed use, many more being in an experimental stage. Only a limited number of these are commercialised and may be found in products marketed worldwide.

Details on the DNA sequences associated with the genetic modification are frequently kept confidential by the GMO developer, referring to protection of their technology against competitors. However, some details are known and described in publicly available databases (e.g. Agbios, 2003), including relevant sequence data (e.g. EMBL, 2003; NCBI, 2003). DNA-based analytical methods are likewise described in a publicly available database (EC-JRC, 2003b). For development of detection methods, detailed knowledge of the associated DNA sequences is a prerequisite, but once sequence information and reference materials are at hand, a detection method based on nucleic acid analysis can be developed rather rapidly (within weeks).

4.3. Specificity of DNA-based analytical methods

PCR-based GMO tests can be categorised into four levels of specificity. The least specific methods are commonly called "screening methods" and relate to target DNA elements, such as promoters and terminators that are present in many different GMOs.

The second level is "gene-specific methods". These methods normally target a part of the DNA harbouring the active gene associated with the specific genetic modification. Examples are the Bt gene coding for a toxin acting against certain insects or the EPSPS gene coding for tolerance against a specific herbicide. Gene-specific methods can provide information about the traits of a present GMO, but they cannot be used to determine whether the GMO is authorised or not, if an authorised GMO contains the gene, because the gene can be used in several independent transformation events. Both screening and gene-specific methods are based on detection of more or less naturally occuring DNA sequences, a fact that significantly increases the risk of obtaining false positive analytical results in tests.

The third level of specificity is "construct-specific methods", which target the junction between two DNA elements, such as the promoter and the functional gene. These methods target DNA sequence junctions not naturally present in nature. However, different GMOs may share several DNA elements, for example both the same promoter and gene, and sometimes even the same plasmid has been used to transform plants (e.g. the two distinct maize GMOs Mon809 and Mon810; Agbios, 2003).

The highest specificity is seen when the target is the unique junction found at the integration locus between the inserted DNA and the recipient genome. These are called "event-specific methods". Unfortunately, even the event-specific methods have their limitations. Crossbreeding between two GMO lines may lead to so-called stacked genes. For example, an herbicide-tolerant GMO can be combined with an insecticide-tolerant GMO. Both sets of functional genes are present in the crossbreed but not necessarily linked, i.e. they are likely to be situated on different chromosomes. Quantitative methods cannot distinguish between the gene-stacked GMO and a mixture of its two parental GMOs. This problem is only alleviated if the test is performed on material from a single organism, such as a leaf or a single kernel of grain or seed, in which case the presence of both target sequences is demonstrated from a single individual that consequently must be a gene-stacked

breed. In the US, this type of hybrid GMO is not regulated if both parent GMOs are authorised. In the European Union, however, gene-stacked crossbreeds require separate authorisation and consequently require quantitation as a single GMO.

4.4. Detection and quantification limitations

The limits of detection (LOD) and quantification (LOQ) are defined as the lowest quantities that can be reliably detected and quantified, respectively. This may sound straightforward, but certainly is not, because the limits of detection and quantification are method-specific, but, at the same time, depend on the sample that is being analysed. It has been customary to report the analytical limits found on pure and unprocessed material, e.g. maize flour. However, products like oil, hydrolysed starch, refined sugar and syrups, and fermented (e.g. soysauce) or sour (e.g. tomato paste) products are characterised by extremely low traces and/or highly degraded DNA. Test reports should provide information about the analytical sample. Despite this fact, most certificates from test-laboratories report the LOD and LOQ of the method on high quality DNA. It is possible to distinguish between three types of detection and quantification limits (Berdal and Holst-Jensen, 2001): (1) the absolute limits, i.e. the lowest number of copies that must be present at the beginning of the first PCR cycle to obtain a probability of at least 95% of detecting/quantifying correctly, (2) the relative limits, i.e. the lowest relative percentage of GM materials that can be detected/quantified under optimal conditions, and (3) the practical limits, i.e. the limits applicable to the sample that is being analysed (taking into consideration the actual contents of the DNA sample and the absolute limits of the method). It has been proposed (Berdal and Holst-Jensen, 2001) to distinguish between the LOD/LOQ of the method and the practical LOD/LOQ of the test sample and to report both sets of values.

4.4.1. Reference genes

Relative quantitation is the same as determination of the ratio of one target to another. In the context of GMOs, relative quantities reflect the ratio of a GMO-derived target to a reference target, usually a species-specific target. The reference target is often a so-called housekeeping gene (preferably a gene with a stable, known copy number and a DNA sequence stable in all varieties of—and unique to—the species).

4.4.2. Genome-related problems

The approximate genome size of crop plants, such as maize and soybean is known. Therefore, it is possible to estimate how many genome copies a given amount of DNA will contain. To get a positive result with a sufficiently high degree of probability, the GMO content must be above a certain limit, i.e. the LOD/LOQ. The quantity of DNA that can be included in a PCR without significant inhibition effect on the PCR is limited (100–200 ng is often the maximum). If this DNA is entirely derived from a single species, the practical LOD/LOQ is determined by the genome size and the maximum quantity of input DNA (template) and can be calculated directly from these parameters. Since the genome size differs considerably within and between plant species (e.g. maize 2.4–5.0 gigabasepairs versus soybean approx. 0.9–1.2 gigabasepairs) the true LOD/LOQ will differ within and among the species. However, if the number of species genomes is determined, e.g. by quantitative PCR, the practical LOD/LOQ can be determined more accurately.

The initially transformed plants are hemizygous with respect to each new transformation event (but not necessarily with respect to the insert or trait) and a diploid (2n) plant would have a relation of copy numbers between event-specific GMO-targets and plant-specific targets of 1:2. Transformed plants are often self-pollinated to yield homozygous (inbred) lines and the ratio of target copy number would shift to 1:1 for the diploid. Hybrids of cultivated plants are very often tetra- or polyploid (i.e. they have four or more sets of chromosomes), a fact that need to be taken into account in relation to cross breeding. Since we often do not know the true level of zygosity or ploidy for the material to be analysed, a high degree of measurement uncertainty is associated with quantitative analytical estimates. Inbred homozygous GMOs are usually crossed with non-GMO varieties adapted for special geographical and climate conditions to yield plant hybrids, which possess both the features of the non-GMO variety and the GMO. Even in a pure GMO, it is therefore possible that the event-specific target can exist in a 1:2 (diploid), 1:4 (tetraploid) or other ratio relative to the plant species-specific reference target.

4.4.3. High-throughput methods and methods for non-authorised GMOs

GM crop production and the number of field trials increase worldwide and seen against the background of the large-scale trade of agricultural goods between countries and continents, a co-mingling with unauthorised GM varieties cannot be excluded. Several cases of GMOs, authorised elsewhere but not in the EU, that were present in batches grown or marketed within the European Union have been reported. Authorisation normally requires a preceeding extensive risk evaluation. Validated methods to routinely detect or quantify the presence of unauthorised GMOs will not be available in the near future, especially if sequence information and reference materials are completely lacking. To overcome this problem, deposition of materials and

information would have to take place on a voluntary basis, requiring a neutral and broadly accepted global institution. The main challenges in the context of GMO detection method development will be to cope with the increasing number of GMOs authorised worldwide and to develop methods for detection, characterisation, and quantitation of non-authorised GMOs. Strategies to this end will have to comprise exhaustive and accessible collection of data on GMO sequences and other data disseminated worldwide.

4.4.4. Reference materials and internationally harmonised analytical methods

At present, only a limited number of certified reference materials are commercially available and there is no reference material accessible for GMOs that are not authorised in the EU. New strategies to improve the availability of reference materials have been explored, including the use of material from contaminated products (Holst-Jensen et al., 2003) and development of plasmids as reference materials (Taverniers et al., 2001; Kuribara et al., 2002).

The above mentioned aspects like ploidy and zygosity are also of high relevance for the selection and production of matrix based reference materials used for calibration because of the direct impact on the quantitation (factor two difference in results if, for example, either a homozygous or heterozygous material would be used for calibration). Effects like DNA degradation, DNA quality and length, and the similarity in behaviour of the reference material used for calibration and method validation and the DNA extracted from a field sample in PCR reactions play an important role for production of reference materials and their foreseen application (Corbisier et al., 2002).

Only few of the available methods have been validated in a collaborative trial according to international harmonised protocols (cf. EC-JRC, 2003b). In the future, methods will have to be harmonised internationally. Several European and worldwide collaborative trials have already been organised and draft European (CEN) and international (ISO, 1990, 1999) standards are already available. Unfortunately, the number of GMOs for which methods are available in these draft standards, is very limited. In this context, the role of the ENGL will be crucial from an EU perspective. A key issue in the context of standardisation of methods will be the determination of criteria for method validation, method acceptance, and test reports. Corresponding efforts are ongoing within a currently constituted working group under the roof of Codex Alimentarius (Codex Committee on Methods of Analysis and Sampling) and ENGL, where minimal requirements for the evaluation of data will be fixed. The new proposal for EU legislation on GMO food and feed takes the need for reference materials and sequence data into account. It is proposed that along with the notification of the product, the notifiers of GMOs have to supply materials and a detection method to the European Commission. However, the "fitness for purpose" conditions are still in the process of being defined. It is absolutely necessary to define, with highly detailed specifications, the types, desired properties and quantities of materials required, the time frame in which materials shall be available, the type(s) of detection methods requested, etc. At present, these issues are being discussed in various fora, including the ENGL.

4.4.5. Reliable identification and quantitation

So far, the genetically modified DNA in all authorised GMOs (plants) is located in the nuclear genome. However, it is possible for a genetic modification to be located in extranuclear genomes as well (e.g. in chloroplasts). The number of extranuclear genomes is not stable relative to nuclear genomes, and some extranuclear genomes are inherited uniparentally, i.e. from only one of the parents. Consequently, any extranuclear location of a genetic modification will create problems in the context of quantitation. Bacteria are microorganisms without a nucleus, and they often possess extrachromosomal DNA (plasmids) that share some features with extranuclear DNA in GMOs. However, plasmids can also be horizontally transferred, i.e. even between different species. Extrachromosomally located genetic modifications may therefore create problems both in the context of identification and quantitation of the GM microorganisms.

4.4.6. Matrix-specific effects and DNA extraction

Although validation in different types of matrices is frequently discussed, this should be seen in relation to validation of DNA extraction methods rather than in relation to the PCR end determination methods. If the extracted DNA is of sufficient quality for PCR analysis in terms of, for example, fragment length and the absence of inhibitory factors, the only effect from the matrix would in theory be linked with the quantity of extracted DNA that can be included in a PCR and this should only have an impact on the practical LOD/LOQ.

However, this is only true as long as the sample is homogeneous and the extraction of DNA from all particles is of similar efficiency. Indeed, each tissue type and plant species differs in chemical composition, resulting in unique extraction and purification efficiencies. The PCR amplification efficiency is clearly depending upon the extraction methods used and it was recently shown that 35S promoter or *cry9C* (or both) could not be detected in 0.1% CBH351 (Starlink) maize extract, whereas endogenous *adh1* gene was detected, depending on the extraction method used (Holden et al., 2003). In the same paper, significant differences in extraction

efficiency between methods were found. The extraction efficiency was also shown to depend strongly on the particle size (Prokisch et al., 2001), which is expected to contribute to bias if, for instance, the GMO material would be mixed with non-GMO material of a different particle size or extraction behaviour. Therefore, appropriate validation of extraction method, as well as precise quantitation and quality measurements of the extracted DNA concentration are crucial.

5. Protein-based methods

Principally, many methods that focus on fractionation, separation, and profiling of proteins and peptides, such as isoelectric focusing, affinity chromatography, and one- or two-dimensional separation approaches might be applicable to the characterisation of GM plant varieties in comparison with the parental non-transgenic line. Unfortunately, the resolution is frequently insufficient or resolved patterns too complex to clearly distinguish a novel GMO-derived protein from the protein pattern of its conventional counterpart. Recent developments in two-dimensional gel electrophoresis significantly improve identification and resolution, but still may generally not be applicable for unequivocal identification of a unique (trans-) gene product unless combined with immunological methods.

In the past 30 years, a wide variety of immunoassay formats have been developed to allow either visual or instrumental measurement of the primary binding reaction between antibody and its target antigen. Conjugates of antibody with fluorochromes, radioactive isotopes, or enzymes are often used for the visualisation of the primary antibody-antigen binding reaction. Direct double antibody (preferred) and indirect triple antibody sandwich ELISA formats have most frequently been employed to detect and measure novel protein produced by GM plant varieties. These methods are applicable to the measurement of bivalent and polyvalent antigens and are referred to as a sandwich assay because the analyte is sandwiched between the solid phase antibody and the enzyme-labelled secondary antibody (direct double antibody) or a second antigen-binding antibody that is bound by an enzyme-labelled anti-antibody (indirect triple antibody).

Today's marketed GM-crop plants frequently possess novel genes that are transcriptionally regulated by either the plant viral 35S promoter from cauliflower mosaic virus (CaMV) or host plant homologous- or heterologous- promoter elements, which are constitutively active in many cases, albeit sometimes with marked tissue and/or developmental specificity. For instance, Event 176 (GM-maize) has been transformed with two synthetic *cry1Ab* genes; one gene is linked to a specific promoter that confers expression in green tissue (maize phosphoenolpyruvate carboxylase gene promoter), while the other is linked to a pollen-specific promoter (maize calcium-dependent protein kinase gene promoter), resulting in expression in pollen. Cry1Ab production was quantified by ELISA in leaves, pollen, roots, and kernels among three genotypes. It was found that gene expression in kernels was below levels of quantification (Agbios, 2003). Consequently, an immunoassay directed against Cry1Ab of Event 176 for commodity testing of kernels might be of limited usability.

5.1. Selection of the antigen

Of considerable importance during the development of any assay is the source of a well-characterised antigen. In the case of GMOs, the antigen is typically a purified protein. This protein can be an enriched fraction isolated from many different sources (e.g. *E. coli*, plants, baculovirus). The purity of the antigen ideally should be $>75\%$; however, suitable antibodies can be developed using less pure preparation. Certain characteristics of the antigen determine the likelihood of successful antibody generation including size, hydrophobicity, and tertiary structure. Particularly, when expressing antigens in prokaryotic systems (e.g. *E. coli*), posttranslational modifications have to be considered, such as protein maturation (e.g. cleavage of signal sequences from preproteins) or glycosylation, which might modulate the immunogenic specificity in comparison to the plant-expressed homologue.

Perhaps the greatest variable associated with immunoassay development is the type of antigen used for antibody production. It is also important to know in some detail what the antibody is specific to. For instance, an antiserum to soy protein was raised by using ''renatured'' soya protein as the immunogen, so in order to be equally reactive to soya protein from a variety of sources, the same denaturation/renaturation procedure had to be applied during extraction (Allen, 1990).

5.2. Validation

The validation of the accuracy of an immunoassay should result in an estimate of the systematic deviation of the measured result from the true value of a given sample. This should be stated as an absolute error for a quantitative immunoassay and as a ratio between false positive and false negative samples for a semi-quantitative immunoassay. Quantitative and semi-quantitative results, if applicable, should be given in units of weight percentage for genetically modified organisms in mixtures with non-genetically modified organisms of the same species. For quantitative assays, it is crucial that the references most closely mimic the character and diversity of a typical sample, such that they are suitable

to determine, for example, compliance with current labelling requirements.

Accuracy of the protein detection method is demonstrated by measuring the recovery of analyte from fortified samples and is reported as the mean recovery at several levels across the quantitative range. Spike and recovery studies should be completed for each protein and each matrix. The results of spike and recovery in certain tissues may differ at different developmental stages of the tissue. Sampling of materials should therefore be consistent with those of the spike and recovery studies, else such studies need to be completed in the relevant range of tissues at different developmental stages.

In 1999, the first international method validation according to ISO 5725 for a specific protein-targeting immunological method for the detection of a genetically modified plant variety was carried out by the Joint Research Centre of the European Commission in Ispra, Italy (ISO, 1998; SDI, 2003). The validated method had been designed as a sandwich enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies against the protein CP4 EPSPS (Lipp et al., 2000).

5.3. Matrix effects

The scope of applications should clearly define the matrices for which the given immunoassay is applicable. If the same extraction procedure is used for different matrices, proof should be given that extraction efficiency is comparable. The scope of application will be reflected in the availability of suitable reference standards.

To evaluate matrix effects, diluted non-GMO containing extracts should be used as the buffer for making the standard curve of known amounts of each GM containing ingredient. If significant interference is observed at the selected matrix concentration (e.g. greater than 10–15% inhibition or enhancement) or if the shape of the calibration curve changes, the standards should be spiked into an appropriate level of non-GM containing extracts in order to guarantee accurate quantitation.

5.4. Quantitation using protein-based methods

Several limitations are envisaged for quantitative determination with protein-based methods. Since expression levels of introduced traits are tissue-specific and developmentally regulated, protein levels in unknown samples hardly can be compared to those in the reference material used. Furthermore, an accurate measurement is only possible if (i) sample matrices are identical to the reference material or (ii) matched standard materials or standards that have been validated for the matrix are available. Comparability with a given reference standard is likewise impaired when a specimen has been exposed to thermal, mechanical, or chemical treatment during product processing. Since existing immunological methods for GMO quantification measure only one analyte (e.g. the herbicide-resistant protein CP4 EPSPS), taxon-related quantitations cannot be carried out. These methods can therefore only be applied to food samples consisting entirely of one taxon (e.g. soy).

6. Ringtrials, standardisation, validation, and proficiency testing

A ringtrial is an organised test including several participants, where the participants are asked to perform a more or less specified test on a set of samples, which may include both known and unknown samples. A validation study is a verification of the performance characteristics of a measurement procedure. A measurement procedure consists of clearly defined steps, such as sampling, extraction, clean up, and analyte determination. A validation may comprise all components of a measurement procedure or, for reasons of reducing the complexity of data interpretation and easier separation of the uncertainty contributions, may be done in a stepwise approach on parts of the measurement procedure. Validation of a method only refers to the steps defined in the method. This means, if only the final determination step is validated, a method comprised of sample preparation and determination cannot be regarded as validated. Likewise, validation for one field of application does not constitute validation for other fields. Validations can be carried out in-house as a single centre study, but critical validation parameters like robustness, reproducibility, and repeatability of a method are preferably evaluated in a ringtrial where the participants are asked to perform a very specific test, usually with predefined reagents, equipment, and programming. The validation study is meant to verify that the specific test is reliable in all laboratories. Consequently, it is important that factors other than those meant to be tested are under control, for example if the determination step to be validated is a specific real-time quantitative PCR method, it shall be performed on the same DNA extracts by all the participating laboratories. If the laboratories are to isolate the DNA, differences in reported results may otherwise be due to differences in the way DNA was extracted or in the quality of the extracted DNA. On the other hand, it also means that the extraction and the influence of possibly interfering matrix compounds and DNA quality have to be validated separately to ensure full applicability of the measurement procedure for a defined and verified range of sample types. Similarly, only laboratories experienced with methods similar to the method

to be tested should participate in validation studies. A proficiency test is a ringtrial, which is meant to test whether the participants are capable of performing the test. In other words, validation is a test of the quality of the method, while a proficiency test is a test of the quality of the participating laboratory. Failure to distinguish between validation and proficiency testing has undoubtedly compromised several ringtrials meant to validate methods. Consequently, several methods are probably better than the results of the validation studies indicate, but on the other side, several measurement procedures have been only partially validated.

Standardisation of GMO detection methods is now taking place on national, European, and global level. In Germany and Switzerland, official methods for the detection of GMO are available. These official methods are published in the "Schweizerisches Lebensmittelbuch" and the catalogue of official methods according to § 35 of the German Food Act.

Standardisation of methods within Europe is under way in the frame of the European standardisation organisation CEN. A working group (WG 11) within the Technical Committee TC 275 of CEN called "Genetically modified foodstuffs" was constituted in February 1999. Five standard methods are going to be worked out, each including a horizontal part (general guidelines for performance) and annexes (specific detection methods proposed by working group members; (CEN, 2001). The five projects are (1) detection of genetically modified organisms and derived products: nucleic acid extraction, (2) qualitative nucleic acid-based methods, (3) protein-based methods, (4) quantitative nucleic acid-based methods, and (5) sampling.

In parallel on a global level, the ISO working group 7 of TC 34 "Genetically modified food products" has taken action since November 2000. The aim is to elaborate ISO/EN standards for GMO detection.

7. GMO detection, new approaches

New methodologies for DNA-based GMO detection have been developed in recent years that aim to improve part of the 'traditional' qualitative PCR and subsequent gel electrophoresis, while the most significant improvement so far has been the quantitative PCR discussed previously. Other novel developments aim to eliminate the time-consuming gel electrophoresis step after the PCR, reducing the (considerable) risk of contamination during the handling of the samples at the same time. An example of such a development is the piezoelectric affinity biosensor that is based on hybridisation of the amplified GMO-specific fragments to immobilised probes on the piezoelectric sensor. The sensing is based on 10 MHz piezoelectric crystals that are sandwiched between gold electrodes (Minunni et al., 2001). The advantage of the system is that no label is required for detection and the application of the biosensor technology may have better perspectives in complex food matrices. Other developments merely aim to develop less time-consuming, less chemical requiring, and more user-friendly systems, such as the lab-on-a-chip developments (Birch et al., 2001). Some of the techniques described below can also be used to detect unintended changes caused by the genetic modification, as discussed by Cellini et al. (2004).

7.1. Microarray technology

The main principle of the microarray technology is miniaturisation. Standard molecular biological or other biochemical methods can be performed on a much larger scale in much smaller volumes. This makes it possible not just to analyse samples for the presence of an individual or a selected group of transgenic or control genetic elements, but to extend the analysis to thousands of probes in a single hybridisation experiment. The basic idea is that (many) selected probes are bound spotwise in array format to a solid surface with each spot containing numerous copies of the probe. The array is subsequently hybridised with isolated DNA of the sample of interest that is labelled with a fluorescent marker. During the hybridisation phase the labelled fragments will associate with the spotted probes on the basis of complementary DNA sequences. The larger the stretch of complementary sequences is, the stronger the bond will be. After the hybridisation phase, the remaining free labelled sequences, as well as the sequences that are only weakly attached to the probes, will be washed off and the array can subsequently be scanned for individual fluorescence intensity of each spot. Data analysis of the resulting patterns and relative intensities will reveal whether the patterns can be attributed to approved GM varieties. For protein-based detection methods, the microarray technology may also be an interesting alternative in (near) future times. In that case, specific antibodies or other types of selective proteins can be bound to the array and coupled to labelled proteins in the samples under investigation.

The 'standard' solid phase array has, however, some important disadvantages. One is that the fluid with the fluorescently labelled fragments is 'static' on the array during hybridisation. Another problem is that the spotting of high concentrations of probes may lead to steric hindrance during the hybridisation phase. Several new array systems are currently in development to overcome these problems. Examples of promising new developments in this area are the electroarray system, where the fluorescently labelled negative DNA fragments are guided to individual spots that are positively charged in order to increase the rate of hybridisation events (Nanogen, 2003). Other systems increase the surface

where hybridisation may occur considerably by using three-dimensional spot structures, reducing the risk of steric hindrance at the same time, e.g. gel-based DNA chips. This system may even be further improved by addition of a pumping system where the fluorescently labelled fluid is pumped up and down through microporous material to further optimise chances of hybridisation actually occurring between complementary strands (Pamgene, 2003). Other systems have also been developed that are based on probes attached to microspheres in solution, i.e. suspension array technology (Nolan and Sklar, 2002), bead array counter (Edelstein et al., 2000).

As GMO detection necessitates the detection of single genetic fragments in the very large pools of genomic DNA fragments, it will be necessary to develop sensitive detection methodologies that enable this in the relatively small microbial genomes, as well as in plant genomes, which can in some cases be large. To this end, either the pool of DNA sequences should be enriched with the fragments of interest, implying a selection of the fragments that actually can be detected, or the (fluorescent) signal should be enhanced after hybridisation. Various approaches have already been developed. Several variants of a system using gold nanoparticles labelled with oligonucleotides for hybridisation and subsequent silverstaining for enhanced detection have been described (Taton et al., 2000; Cao et al., 2002). Another signal amplification method, which can be directly applied on genomic DNA samples, is represented by the (licensed) Invader™ technology. At the basis of this type of assay

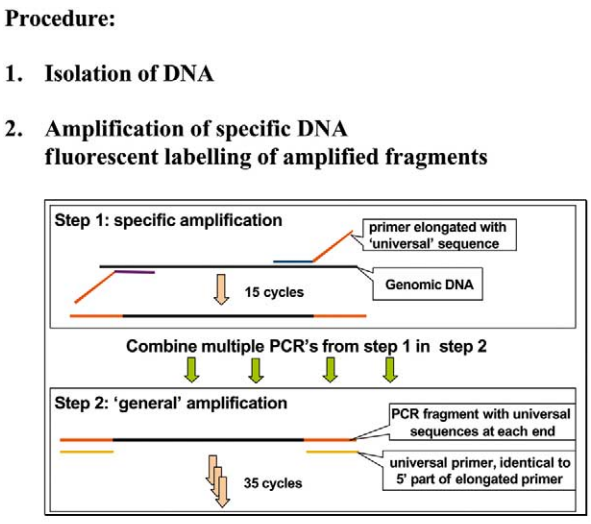

Fig. 1. Procedure for preparing samples for microarray analysis.

are two probes, which bind to the target DNA. One probe is called the "invader", the other "extended primary probe". The overlapping regions are recognised by an enzyme and cleaved. The released flap binds in a secondary reaction to an artificial oligonucleotide (FRET-cassette), which includes a fluorescent-dye and a quencher molecule. After hybridisation, the dye is set free by enzymatic cleavage and a fluorescent signal can be detected. One flap induces the cleavage of many FRET-cassettes and thereby enhances the signal considerably (e.g. Hessner et al., 2000). The assay is principally suited for high-throughput and requires only nanogram to sub-nanogram amounts of DNA.

It is more than likely that developments in biotechnology worldwide will necessitate such powerful methods for adequate detection and identification of GMOs, whenever such analyses are required by operative (EU) regulations. The microarray technology also has the theoretic potential to detect unauthorised GM varieties that have any similarity with known (parts of) genetic constructs. Microarray systems are now under development that screen for transgenic (border) elements in approved GM varieties as well as for unique control elements for the relevant species or possible sources of contamination (GMOchips, 2003; Rudi et al., 2003; Kok et al., 2002; Aarts et al., 2002). Fig. 1 depicts the work-up of DNA samples prior to microarray analysis for the presence of DNA fragments from GM soy and maize as depicted in Fig. 2. In this way, all known and/or approved GM varieties will show a specific pattern on the array and aberrant patterns may indicate that unapproved GM varieties are present in the sample. Further analysis on the basis of the aberrant pattern and the underlying DNA sequence(s) will then be necessary to determine the nature of the (GM) contamination. With increasing numbers of probes on the array appropriate data analysis instruments will become increasingly important. The quality of available software for microarray data analysis is, however, continually improving and detection and identification array initiatives will largely benefit from all the efforts that are currently ongoing in the area of gene expression profiling where analysis of large data sets is already becoming a matter of routine.

7.2. Mass spectrometry

In recent years, mass spectrometrical methods based, for example, on the MALDI-TOF MS (matrix-assisted laser desorption/ionisation- time of flight mass spectroscopy) principle have gained more and more importance in analysis of larger biomolecules like proteins, but also oligonucleotides. In MALDI, the analyte is embedded in an UV absorbing matrix in vacuum on a carrier between electrodes. Subsequently ultraviolet laser light is applied. UV-energy is absorbed by the matrix and

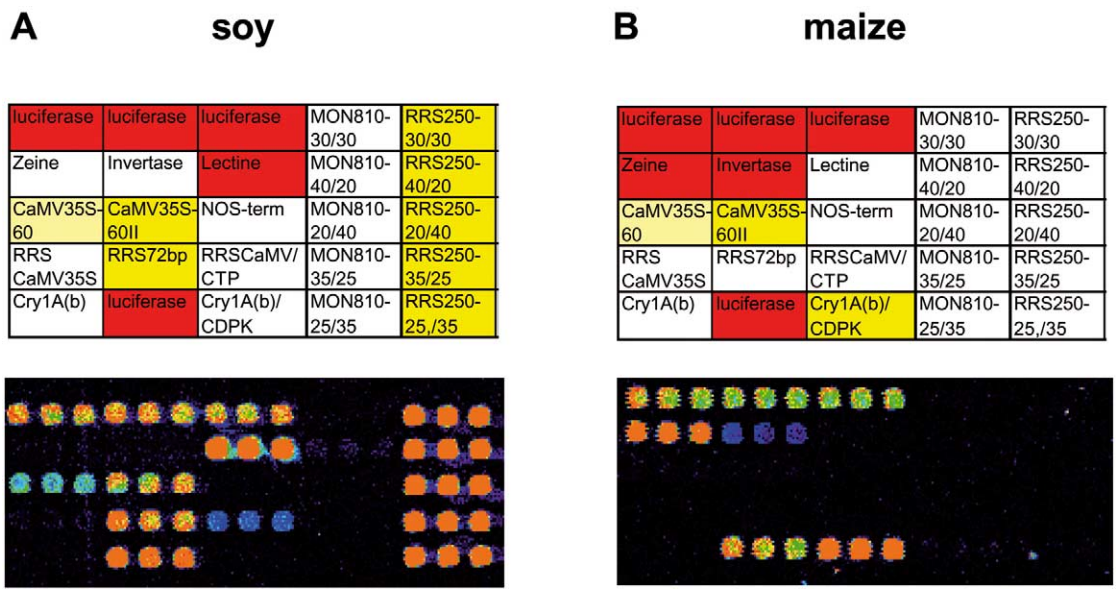

Fig. 2. Example of the analysis of GMO-containing samples using DNA microarray technology. A, soy sample containing a low percentage Roundup Ready Soy (2%); B, maize sample containing a low percentage Bt-176 maize (1%). Each probe is represented by 3 spots placed next to each other on the microarray. Yellow box: GMO-specific probes; red box: control DNA for positioning microarray and hybridisation efficiency (luciferase) and for plant [specific for soy (lectin) or maize (invertase and zein)].

also carried over to the sample (polymer) such that it will be ionised. The ionised molecules move towards the oppositely charged electrode and enter the flight tube towards the detector. During the time of flight (TOF) until detection, the molecules are separated according to their mass to charge ratio. The technique has been successfully applied in genotyping of single nucleotide polymorphism (SNP) of genes. Since usually an amplification of the target by primer elongation is the preceding step, the analysis comprises of two steps where mass spectrometry is restricted to the final detection. Experience with GMO detection is not available yet, but a future application may be coupling of mass spectrometry with other techniques like SPR (see below).

7.3. Surface Plasmon Resonance (SPR)

SPR uses thin-layered metal films (plasmon) on a sensor chip (usually gold-coated) to which the biomolecules (protein, DNA, oligonucleotides) are bound. The surface is rinsed with fluid that contains a binding partner to the surface-attached molecules. Biospecific interaction between the particles is studied by polarised light, which is reflected from the plasmon surface. If molecules from the fluid and those bound to the chip get linked to each other, the reflected light intensity is reduced. The size of the change in SPR signal is directly proportional to the mass. The advantages of the technology are: (i) it is not necessary to have highly purified components, (ii) quantities below microgram can be analysed, and (iii) analysis and detection are done by one step in a real-time procedure. First results using this technique for the detection of Roundup Ready™ soybean have been reported by immobilising biotinylated PCR products or target oligonucleotides on the chip and hybridising them with respective probes (Feriotto et al., 2002). The potential of the method in the field of GMO detection and identification has to be evaluated.

8. Achievements and challenges in GMO detection

The results of recent research projects and activities in the framework of the ENGL have demonstrated that several of the major challenges identified three to four years ago, can be or have been solved. For instance, detailed characterisation of the transformation event at the sequence level is possible and has been demonstrated for a substantial number of GMOs, in particular within the EU-funded QPCRGMOFOOD project (QPCRGMOFOOD, 2003). Such characterisation allows for more detailed genetic maps, including information that is essential for development of event-specific detection assays, as well as details—and nature—of insertion loci and potential unintended partial inserts and rearrangements of the host genomic DNA. However, joint presence of event-specific sequence motifs will be observed in so-called gene-stacked GMOs, i.e. the offspring of hybrids produced when two or more GMOs are crossed. Unfortunately, event-specific detection and quantitation methods will not be able to distinguish between the gene-stacked hybrid and a mixture of the parental GMOs. No alternative detection methods

capable of solving this problem are foreseen in the near future.

Also identification of 100% of an ingredient based on DNA sequences unique to a single species, where the DNA sequence is stable in all varieties both in terms of copy number per haploid genome and in terms of alleles, has been achieved. These DNA sequences serve as suitable and necessary references for PCR based quantitation, including determination of the LOD and LOQ of GMOs in test samples. Notably, in the QPCRGMOFOOD project, several candidate genes presumed to behave as described were found to vary at the allele and/or copy number, and were consequently rejected for future use as reference sequences. A strategy was developed and proved very useful to determine the reliability of candidate reference sequences (Hernandez et al., 2001). Similarly, homo- and heterozygous, as well as di-, tri-, and polyploid lines will yield divergent quantitative estimates of the GMO content using any known molecular detection method. It is also very unlikely that the DNA content per weight unit of, for example, grains is the same when produced from different lines, or when they have been subjected to different handling conditions (e.g. relative water, starch and protein content may differ). It should be noted in this respect that it is believed that the analytical steps from sampling to extraction of analyte (DNA or protein) account for most of the measurement uncertainty in the final quantitative estimate of the GMO content.

Recently developed PCR-based quantitation methods combine event-specific and reference sequence-based results into estimates of GMO content, which are very accurate and highly reproducible between laboratories provided with the same starting material. This means that the measurement uncertainty associated with the final quantitative estimate is primarily caused by steps preceeding the quantitative PCR and the underlying genetics. Consequently, there is only limited room for further improvement of PCR-based quantitation. At the same time, validation of PCR-based detection and quantitation methods is time- and resource-consuming. By separate validation of reference sequences—and GMO—specific assays using calibration curves based on target sequence copy numbers, the workload associated with validation of assays for new GMOs is reduced by almost 50% relative to conventional method validation assays. However, this simplification requires similar behaviour of the DNA used for calibration compared to the DNA extracted from the field samples. This also allows for a more flexible use of reference genes in combination with specific assays for each GMO in test laboratories, and can reduce the workload in the test laboratories significantly.

Informative reporting is very important for any analysis report and also in the case of GMO detection. Traditionally, certificates from test laboratories operate with method LOD and LOQ for each GMO, determined under exceptionally optimal conditions with, for example, certified reference materials. Unfortunately, the method specific LOD/LOQ is not applicable to processed or composite products, and may therefore give a misleading impression of the GMO content, in particular when the certificates report that no GMO was detected. By using a copy number-based approach, it becomes possible to determine the LOD/LOQ for each GMO in each test sample and to express these limits much more reliably (and still as relative values, i.e. as percentage). This will certainly improve the value and reliability of test certificates, if implemented.

Multiplexing of qualitative analyses is a necessity, because the number of GMOs to be tested for is already high and steadily increasing. Significant progress has been made within the EU-funded QPCRGMOFOOD—and GMOchips (GMOchips, 2003)—projects, as well as national projects, and further progress is expected. Multiplexing requires extensive testing of compatibility of PCR primer pairs, etc. (Kok et al., 2002), and PCR-independent alternatives are currently investigated.

9. Differentiation in the GMO sector: traceability, segregation, and Identity Preservation (IP)

A current tendency in food production is the differentiation of products on the basis of a wide variety of characteristics. As a general implication of such differentiation, the commodity-based system, where grains from different origins are considered sufficiently similar and marketed at similar prices, would shift to a more "brand-like" distribution.

Traceability, segregation, and Identity Preservation (IP) represent systems that enable producers to differentiate to various degrees and with various methodologies among products with dissimilar characteristics. In the case of GMOs, the differentiation is primarily between GMO-derived and non-GMO-derived products. In ISO 8402, traceability is defined as "the ability for the retrieval of the history and use or location of an article or an activity through a registered identification" (ISO, 1994). When applied to food production, the multifaceted concept of traceability can be defined as the process to map out the overall chronology of the product (raw material, ingredient, or foodstuff) by tracing its origin and history through a registered identification.

In the food context, clear definition and provisions for traceability are put forward at European level in Regulation 178/2002, articles 3 and 18 (European Commission, 2002). With relation to GMOs, the proposed T&L Regulation provides the following definition for traceability: "the ability to trace GMOs and products produced from GMOs at all stages of their placing on the market through the production and distribution chain".

9.1. Scope of traceability in food

In general terms, the target of traceability systems is to guarantee the differentiation among foods with different attributes. Traceability systems represent instruments to provide such differentiation in a reliable and documented manner. Differentiation of the attributes are often a matter of "confidence" in the sense that the consumer cannot perceive whether the products actually encompass that attribute (Golan, 2002). Confidence attributes are both content attributes, in relation to properties not perceivable but ascertainable, and process attributes, for which neither consumers nor testing can prove the difference. The property of a food to be not GMO-derived is a confidence attribute being a content or a process attribute, depending on the possibility to detect protein or DNA derived from the genetic modification.

The aims of the traceability systems are diverse, depending on the different interests of the parties involved and the characteristics of the attributes to be separated; the most relevant goals, spanning from marketing to risk management, are to:

1. Make a distinction among products with different quality markers
2. Trace back for control purposes, with both quality and health as ultimate targets (withdrawal in case of negative effect)
3. Improve supply-side management
4. Provide data for epidemiological studies
5. Achieve transparency toward consumer

The main aims of traceability described above outline a gross distinction between voluntary (quality purposes) and mandatory traceability (safety purposes) systems, depending both on the ultimate scope and on the attribute under consideration. Different levels of the traceability systems can be established depending on the ultimate scope of the attribute to be traced and on the desired level of accuracy and reliability.

9.2. The application of traceability to the GMO sector

While traceability represents a rather well established and accepted concept in food production, its application to GMO supplies is still raising controversies and debate. Provisions for traceability "from farm to fork" are considered in current (Directive 2001/18) and proposed (T&L) EU regulations, as well as at the international level, such as in the Codex Alimentarius framework.

The five goals of traceability for the food sector in general outlined above could all fall within the scopes of traceability in the GMO area. The record-keeping procedures in GMO traceability represent a tool to facilitate and document a factual differentiation between GMO- and non-GMO- derived products, to help labelling and to individuate responsibilities among operators. The possibility of effective recall procedures through product tracing may prevent excessive economic losses and/or brand damage.

From the time when the first generation of GMOs arrived on the market, the request for non-GMO-derived products has increased in several parts of the world, such as in Europe and Japan, and traceability of GMOs is mainly related to the exclusion of GMO-derived goods from conventional or organic products. In addition to the above market-driven rationales for traceability, other motivations were also invoked, such as the facilitation of postmarketing surveillance of individual GMO-derived products.

In the near future, traceability may also become relevant for the differentiation of high quality GMO-derived products (such as the second generation of GMOs addressed to consumer needs) for which a higher price will be paid as a quality grade food. In that case, the payment of a premium for differentiated products will be consequential.

9.3. Traceability in current and proposed legislation

9.3.1. EU Regulations on traceability

The need for traceability systems in the area of GMO-derived products originated from the indications in Regulation 258/97, where labelling requirements have been set for GMO-derived products to enable consumers to make a choice, even though in this regulation no clear reference to traceability is made.

As mentioned above, Directive 2001/18 clearly introduced the principle of traceability for GMOs, requiring that Member States ensure traceability at all stages of the placing on the market of GM products. In this regulatory framework, provisions for traceability are seen as a valuable tool for environmental and health protection and monitoring, while they are regarded as an important prerequisite for labelling, as well as for withdrawal in case of an unexpected adverse effect to human health or to the environment. However, the Directive does not provide details on definitions, target, and procedures for implementation of traceability in the GMO sector, but it calls for a horizontal Regulation on traceability to complement the Directive. These relevant aspects of traceability are detailed and clarified in the T&L and F&F proposals.

In particular the T&L proposal lays down the following requirements to implement traceability of GMOs at all stages of their placing on the market: "Operators shall have in place systems and procedures to identify to whom and from whom products are made available". In addition, "Operators shall transmit specified information concerning the identity of a product in terms of the individual GMOs it contains or whether it is produced

from GMOs''. Whatever the final provisions in the T&L and F&F Regulations will be, their adoption will have a considerable impact on the food and feed chains, both at European and at international levels. The more marked the differences in regulation of GMOs worldwide in terms of provisions for labelling and number of authorised events are, the larger the impact on the trade of food and feed products will be; this will in turn affect implications and costs of traceability at the country level.

9.3.2. Regulatory discrepancies between EU and US

Negotiations on international systems for GM product tracing under the auspices of international organisations, such as the United Nations Codex Alimentarius Commission, are proving difficult. Official positions of especially the European Union and the United States diverge on which objectives justify mandatory traceability provisions. In contrast to the EU, the US has serious concerns regarding mandatory product tracing systems for reasons other than food safety. The US strongly opposes mandatory measures to support product labelling, consumer information, or identity preservation of a product. According to the US, products tracing should only be considered where it is necessary to protect the health of consumers, to meet a food safety objective, or to manage an identified risk. Tracing requirements should be scientifically based on risk assessment. In practice, the US government establishes food safety performance standards that food production and processing plants must meet, that then are continually verified through inspections. Negligence in carrying out HACCP (hazard analysis and critical control points) plans results in regulatory action.

Furthermore, opinions differ on the extent to which mandatory traceability provisions should be verifiable. In consequence, there is disagreement on what product information should be recorded, and on which role governments and international organisations should play in coordinating international systems for traceability. Agreement exists on traceability systems relying on the 'one-step-forward-one-step-back' principle, by which record keeping at each point in the food production and distribution chain is required only one step forward—it needs to be recorded where the products are sent to, and one step back—it needs to be recorded where products were obtained from. In the US, this system is voluntary. The industry's incentives to apply such a system may be three-fold: (i) to facilitate traceback and withdrawal of food in response to concerns on safety or quality; (ii) to differentiate and market foods with undetectable quality attributes; and (iii) to improve supply side management. This voluntary system in general does not make full traceability back to the farm of origin feasible for bulk commodity supplies like grain or oilseeds. The large volumes handled and bulk-blending practices complicate identification of the farm of origin, unless the grain is identity-preserved for its quality attributes. Nevertheless, both the EU and the US contribute to efforts to develop a system for unique identifiers for transgenic crops under the auspices of the OECD (Organisation for Economic Cooperation and Development).

The dual objective of provisions for traceability as laid down in Regulation 178/2002 on the General Principles of Food Law is to facilitate targeted withdrawals related to product safety concerns and to provide appropriate information to consumers or control officials, where necessary (European Commission, 2002). The US administration, on the other hand, argues that effectively enforced food safety performance standards are preferable to standards relating to product tracing processes. It asserts that market forces, specific government performance standards for food safety, and liability provisions suffice as a basis for a self-organising traceability system that links the divers players of the agro-food production chain. Upon publication of the EU's traceability laws, the US administration protested that compliance costs to US operators with the European traceability and labelling regime for GMOs would amount to 4 billion US dollars per year. The Consumer Federation of America, on the other hand, does not concur with the official position of the US administration. The Federation strongly advocates mandatory labelling to help the technology to gain the public trust that is essential to fulfilment of its promise.

The attitude of the US administration with respect to mandatory registration and record-keeping of entities of the agro-food chain, however, appears to change with the new awareness of vulnerability triggered by the terrorist attacks of 11 September 2001. This is evidenced by provisions to enhance the security of the US food supply in the 2002 Bioterrorism Act (FDA, 2003). The Act contains requirements relating to registration of private entities, improvement of inspection procedures, and new information systems based on notification of imports of food products and record keeping in the agro-food production chain.

9.4. Systems for the differentiation of GMO— and non-GMO—products

The differentiation of GMO and non-GMO products can be advantageous for two different ultimate scopes: to keep the content of undesirable GM material below the allowed threshold in the non-GM food production chain, or to keep and commercialise GMOs separately due to their high added value such as in consumer-directed second generation biotech products. This section is mainly devoted to the systems aimed at the first scope. However, most of the considerations below are valid also for differentiating high-grade GMOs.

Also the non-GM products can represent a value-enhanced product for which a premium has to be paid: the stricter the degree of differentiation from GM products, the higher the price would be. The current costs of "keeping apart" non-GMO-derived- from GMO-derived- products are already paid at all stages of food production, but this is apparently not yet perceived by- or charged to- the consumer: in the long run consumers will probably have to pay a higher price for non-GMO-derived food products.

A controversial issue at European level is represented by the threshold(s) for the compulsory labelling provision of GMO products. The threshold level greatly influences procedures to be adopted in order to achieve differentiation between GMO- and non-GMO-derived products. Such differentiation can be practically achieved mainly through two apparently similar systems: the segregation and the Identity Preservation (IP) system. Definitions for those systems have already been given (European Commission, 2000d; Buckwell et al., 1998; House of Commons, 2000; Lin, 2002a), but only few of them agreed in term of principles and strategies they encompass. Basically, those definitions include the following principles:

9.4.1. Segregation system

According to Lin's definition, this process implies that crops or lots of ingredients are maintained physically separated in all steps of the supply chain systems at various levels of precision to avoid commingling, while the segregation system starts at farm level. Containerised shipment is usually not necessary. However, cleaning of the facilities such as augers, as well as transportation and storage facilities, is required at all steps. In addition, testing to check for the presence of GMO-derived materials is required throughout the supply chain in such a process, whenever the product changes hands, which makes the whole process rather costly. In principle, this system does not necessarily imply that an accompanying traceability system is present (European Commission, 2000d).

In other words, segregation is defined as the production-handling-distribution process that requires separation of crops to avoid commingling during loading and unloading, storage, and transportation.

9.4.2. Identity Preservation system (IP)

Identity Preservation is an amalgam of processes, protocols, systems and initiatives that have been in place for many years, mostly under the banner of quality systems. Originally IP, HACCP, traceability and related systems were aimed at removing the risk of contamination, ensuring certainty of quality characteristics and improving the basis on which healthy food is produced, avoiding the legal and economic consequences of a breach of contract, etc.

Conceptually, IP is the creation of a transparent communication system that encompasses all participants in a supply chain. Buckwell et al. (1998) define IP as a system of crop management and trade which allows the source and/or nature of materials to be identified. This process guarantees that certain characteristics of the crop or lots of food (such as the non-GM origin) are maintained "from farm to fork". According to many authors, this is achieved by means of containerised shipment. In this way, the need for additional testing when the commodity changes hands is decreased. In other words, the ideal IP system will be one that contains information on the how, what, when, where, who, and why of a particular product.

Similar to traceability systems, IP provides means by which it is possible to backtrack through the system to identify where contamination may have occurred.

9.4.3. Segregation vs. IP systems

Despite the distinct definitions of segregation and IP described above, a clear distinction between the two processes is frequently not made, because there is no marked border between them. In practice, the terms are often being used interchangeably.

Segregation and IP could be considered as a continuum in the process of differentiation of non-GMO-derived- and GMO-derived- products, both starting at the farm level. An alternative could be to use the term differentiation, the extent of which depending on the requested degree of purity. On the basis of the considerations described above, the term traceability could be useful, implying both the two following meanings:

The systematic recording of the presence of GMOs from creation through marketing
The practical approaches/systems to achieve differentiation between GMO- and non-GMO- products.

9.4.4. Factors linked to traceability

The GMO scenario is continuously evolving and therefore the current implications, needs, and costs of traceability most probably differ from those in the long term. Traceability systems are strictly linked to a high number of peculiar aspects, many of them dramatically influenced by socio-economic factors. Among these factors are:

- the extent of GMO crops cultivated worldwide, including the possibility of GM cultivation in Europe. In 2001, over 120 million acres of transgenic crops were cultivated globally (in US: 60 million acres transgenic soybean and over 25 million acres transgenic maize); in Europe no transgenic crop is cultivated commercially except for 62,000 acres of maize in Spain (James, 2002).

- farming practices (location around the world, size, facilities), storage (equipments, size, location), transportation, and marketing that are peculiar to GMOs and non-GMOs. In this respect, marked differences exist between European and non-European countries, especially when the volumes of produced and exported crops are particularly large, as in the US. This latter aspect significantly affects the production chain, since large volumes could stimulate the shifting to dedicated equipments. On the contrary, small volumes could support the trend to verticalisation of the whole handling and processing, since concentrating more than one step in the same point could reduce the costs of differentiation. This, as well as many of the aspects mentioned above could in turn influence the premium that the consumer will be willing and/or will have to pay for non-GMO-derived products.
- the consumer acceptance of GMO-derived products and the consequent extent of the demand for non-GMO-derived products. The surveys of the Eurobarometer have demonstrated the scarce acceptance of genetically modified products by the European consumers (e.g. Gaskell et al., 2003).

In the light of these considerations, any absolute statement related to the implication and cost of traceability can be neither permanent nor valid for each country. Several authors and organisations have published valuable studies on the issue of implication of traceability in different simplified scenarios (Price et al., 2002).

9.4.5. Implications for traceability in each step of the food chain

The demand for traceability of GMOs has its particular implications for each step of the food chain. Moreover, these implications should be considered in a global perspective for the GMO sector, mostly since GMO maize and soybean varieties have been largely adopted in the US, and a large part of these US products is exported worldwide.

9.4.5.1. Farm level. Effective IP begins at farm level and two main aspects should be taken into account. First, pollen drifts can lead to the unintended presence of contamination of non-GM crops. Recommendations were recently given by the UK House of Commons (2000). Buffer zones may help to minimise GMO contamination from pollen drift, but it remains a serious problem for effective crop IP (Thomas, 2000). Pollen drift is less for self-pollinating crops like soybeans than for maize. Second, harvesting demands attention especially when harvesting both non-GM and GM fields. Therefore, the crucial points of IP/traceability at the farm level are the use of non-GMO starting seeds or planting, as well as the documented agricultural practices for planting, field crop management, harvesting, transportation, and storage of non-GMO.

9.4.5.2. Elevator level. Elevators must also develop stricter control over handling procedures in order to maintain segregation, the characteristics of the elevator greatly influencing implications and overall (administrative and contracting) costs of traceability. In this respect, it should be underlined that the elevator structures are mostly rather different in US and in Europe. Especially for elevators that operate with high volumes of grains, differentiation between GMO— and non-GMO—derived grains will slow the turnover of the elevator considerably, lowering the profits at the same time.

Also the location of the elevator can influence the implication and costs of traceability, since elevators close to the river can better avoid commingling as they can load directly on the vessels, while the inland elevators have to perform a higher number of loading and unloading operations with higher possibility of commingling (Lin, 2002a).

Possible strategies to facilitate segregation can be: (i) specialising different locations for non-GMO storage, or (ii) acceptance of non-GMO and GMO products on different days to enable regular cleaning activities.

9.4.5.3. Transportation, storage, and distribution. Transportation implications will vary in relation to the maximum amount of accepted GMO (threshold value). A threshold limit of 1% could double the cost of transportation for soy (Lin et al., 2000), since the differentiating system implies containerised shipment, while, according to the North American Grain Exporters Association, a tolerance level of 5% or higher could not affect significantly the cost of transportation. However, the pattern of transportation will become more complicated due to the coexistence of more than one GMO-event in various crop species.

In addition, documented commodity storage and transportation management practices for handling of non-GMO crop is particularly crucial in cases for which changes of hands are manifold.

9.4.5.4. Transformation of raw materials into ingredients. The characteristics of the industry are also of relevance in implementing differentiation and traceability of non-GMO- from GMO- derived commodities. Besides the same inbound costs (like the elevators), processors must purge, or, in extreme cases, stop and clean the processing plant when changing the type of grain being processed. Processors are subjected to essentially the same risks as the country elevator owners, but the potential cost of processor's risks is higher due to the added value of the product.

9.4.5.5. Cleaning procedures. Requested accuracy and, consequently, costs of cleaning procedures are strictly dependent on the threshold level. The more stringent the tolerance level is, the more costly and crucial the cleaning procedures are.

The cleaning of machinery (combine, country elevator, conveyor belt, etc.) and transportation vehicles (truck, train, ship, etc.) is very important in this respect. It was shown that the time needed for cleaning activities is relatively little, but, in practice, it may be difficult to comply with rigid cleaning protocols during the busy time of harvest (cleaning and flushing costs) (Van Rie, personal communication).

9.4.5.6. Audit and certification. All relevant steps in the chain must be subjected to internal auditing of compliance with the traceability program requirements and supported by verification by independent auditors. Certification of traceability handling must be provided for the product.

9.4.6. Factors affecting the feasibility of traceability

Along with the implications outlined in the previous section, implementation of traceability provisions have to face with many additional challenges related to the state of art of both regulations and diagnostics. In particular, a considerable number of factors influence the feasibility of the implementation of provisions of current and future legislation. The most relevant are herein considered in some details.

9.4.6.1. Criteria for unique identifiers. A prerequisite for the implementation of traceability is represented by the establishment of a system for development and assignment of unique identifying codes to GMOs as requested in the proposal of Regulation T&L. The need for specification of the GMOs' identity is required by the Cartagena protocol on Biosafety, article 20, paragraph 1, which established a Biosafety Clearing-House (Convention on Biological Diversity, 2000) and work is ongoing at OECD level on this topic. Adoption of compatible systems would facilitate commercial transactions.

9.4.6.2. Control methodologies. Reliability and costs of the two steps of the control methodologies (sampling and testing) play a crucial role in the traceability of GMOs. The technical aspects of both methodologies have already been discussed at length. In general, type and frequency of control methodologies depend on the extent and reliability of differentiation to be reached and on the matrix to be analysed.

Testing implications and costs depend on the step of grain handling/processing in which the test is performed. Several studies (Lin, 2002a; Gustafson, 2002) have been carried out to evaluate the costs of testing at different levels and the results are in some cases rather different. The PCR testing can take from 1 to 10 days, costs from 100 to 400 Euros and provide a sensitive and reliable response for many traits. The on-site ELISA test can take 2–8 h and costs 10 Euro per test. An ELISA dipstick test (yes or no answer) can take few minutes and costs 3 Euros each. However, current PCR- and ELISA- methods require one test for each event and therefore for maize, many tests can be required in order to guarantee it as a non-GMO-derived material.

Since the number of the events are continuously increasing, this will greatly increase difficulties in testing and, in turn, traceability. In this respect, it is foreseen that the testing methods will, as mentioned before, have to evolve towards a multi-event system, to control any GMO-related traceability system.

9.4.6.3. Threshold limit. Since the detection limits of the current testing methodologies are very low, the threshold limit for unavoidable contamination represents one of the most critical points for traceability. The more stringent the limit is, the harder is the task of traceability. In addition, the threshold level should keep into consideration the variability associated to the analytical steps (both testing and sampling). Therefore, threshold level for adventitious GMO contamination can greatly influence the economy of traceability (practical differentiation and record-keeping) and influences all parts involved in the food production. The lower the threshold level for the adventitious presence of GMOs in the food production chain, the higher the costs of implementation of a "GMO-free" IP system will be. Eventually, the costs of such a system may become prohibitive, which, in turn, will also affect the consumers' freedom of choice.

9.4.6.4. Economic consequences of the implementation of IP. The implementation of a suitable IP system implies a substantial increase in the overall costs. By summarising the different costs along the production chain, an estimation of the total costs of IP can be given. According to several studies (Lin, 2002a,b; House of Commons, 2000; Lin et al., 2000) these estimations range from 5 to 25 Euros per tonne, depending on the different grains and the applied IP systems. Thus, IP would increase the grain price by 6 to 17% compared to the farm gate price.

The mentioned costs are the result of a static approach. However, it is likely that the costs can also increase as a result of the dynamics of the various markets. In addition, changes in costs imply changes in prices. This can have repercussions for the demand, which varies between different production chains and depends on the price-elasticity of the demand. The higher the price-elasticity is, the more than proportional changes in demand will occur. This means that a price increase will result in a more than proportional decrease of the

demand and additional costs cannot be transferred to the following stages. Equally, elasticity can be distinguished for the supply price. A less price elastic supply means less than proportional changes in relation to the changes in price. Price changes for a specific product can also result in a switch towards a substitute, i.e. substitution elasticity.

Also agricultural price policy measures can have an impact on the transmission of additional costs of IP. Controlling price measures can limit the transmission towards the consumer or, on the other hand, can limit the implementation of innovations. The factors mentioned above concerning additional costs, the effect of demand and supply, market structure, and agricultural policy are important determinants for the implementation of IP systems.

Producers will face a basic choice between handling non-GMO-derived products at a higher price or conventional products at a lower cost. Without definitive detailed legislation for labelling and traceability, it is rather difficult to predict the cost for implementing the future provisions for traceability.

10. Conclusions

10.1. Regulatory aspects

The current situation with different authorisation and labelling regulations for products consisting of—or derived from—GMOs in different countries or trade areas is confusing and hampers international trade. Global harmonisation of these regulations is therefore urgently needed. Only harmonisation of national requirements for GMO-derived products will lead to increased transparency with respect to the international (GMO-derived) food supply chains. Moreover, harmonised data requirements that are supported by the relevant stakeholders are essential for adequate maintenance of current and future GMO regulations and will lead to internationally accepted analytical methods and reference materials. At this moment, there seems to be a difference in the preference for GMO detection strategies between the EU and the US, with the EU focusing on DNA-based methods, while the US are more concentrated on protein-based approaches. It is, however, possible that DNA- and protein- based methods may give different quantitative results depending on the matrix under analysis.

10.2. Sampling strategy

Sampling is probably the most crucial step in the diagnostic procedure. Among other relevant factors, an appropriate sampling plan also depends on the parameters of the detection method used. Several reliable sampling plans already exist, but none of them have statistically based concepts that are fully adapted to the issues of GMOs or of GMO-free production.

10.3. Threshold level and maintenance

It is considered unavoidable that non-GMO- and authorised GMO- derived raw material batches are regularly mixed, e.g. as a result of field trials or resulting from mingling of the different varieties during transport of especially bulky products. This mingling is practically very difficult, if not impossible, to avoid and this forms the rationale behind the establishment of the current 1% threshold level for the labelling of GM varieties in a GMO-free bulk.

There are, however, technical constraints and uncertainties related to the maintenance of this threshold level, including the interpretation of it in terms of units to be compared (DNA/DNA or weight/weight); the need for reference materials and GMO event-specific sequence information; the issue of 'stacked genes' and unauthorised—including unknown—GMO events; and the increasing number of GM varieties being tested in the field and/or released within the EU and abroad. With regard to multimethods to detect multiple GMO events at once, developments in the area of the microarray technology may be significant in this respect, but will need to be taken further before fulfilling their promise.

10.4. Traceability

Appropriate traceability and segregation systems may reduce the necessity for stringent testing schemes. Besides the administrative burden of the documentation itself, the possibility to detect deviations in GMO contents from those documented in traceability systems may, however, require additional measures such as storage of backup samples and additional testing. In addition, measured GMO content in processed materials may not always reflect the real GMO content in the raw material. This may affect the efficacy of the analytical control options in traceability systems. It is clear that threshold limits strongly influence the necessary separation practices aimed at meeting those limits. The lower the threshold limit is, the stricter and more costly the separation strategies will have to be. Moreover, it should be emphasised that an appropriate traceability strategy for all GMOs in the food production system will require entirely new labelling and information transfer systems.

10.5. Outlook

In general, it can be concluded that there are many technical and political issues to be solved before all GM varieties can be detected and identified in a reliable way

in all matrices in the immense diversity of the current food supply. Important steps have been taken in the last few years. Qualitative and quantitative GMO detection methods have been developed, while initiatives were set up to exchange information on GM varieties and the related detection and identification methods within Europe and worldwide. An important step in this respect has been the establishment last year of the ENGL (European Network of GMO Laboratories). The challenge for regulators will be to combine the consumers' rights to reliable information on food products in the market with the necessity for a safe food supply, taking into account the technical limits to detect, characterise, and quantify any individual GM variety, approved or unapproved, in any matrix of choice.

As for traceability and differentiation, difficulties in their implementation will be strongly linked to the threshold level for the mandatory labelling of GMOs. The lower the threshold, the more difficult the implementation will be. Also the number of authorised and non-authorised events will affect the implementation of traceability.

Acknowledgements

The authors would like to thank Dr. A. König for her contribution to the paper and Mrs.Dr. A.M.A. van Hoef for her illustrations. Part of this work has been funded by the European Commission's thematic network ENTRANSFOOD.

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