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Towards detection of unknown GMOs

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20.1 INTRODUCTION

Increasing technological competence and capacity has gradually facilitated the development of genetically modified (GM) organisms (GMOs) such as plants (GMPs). The scope of developing GMOs varies, and developments may be motivated by anything from scientific interest, via an intention to solve a particular problem, to expected commercial profit. Do-it-yourself bioengineering (biohacking) and even intended dual use (a term referring to hostile/non-friendly usage) of gene technology can no longer be excluded, although the latter is usually perceived as very unlikely because other simpler and more efficient alternatives exist (Holst-Jensen, 2008). It is, therefore, complicated to generate a complete overview of ongoing and recent developments in GMO technology. Technological developments may further reduce the visible genetic fingerprint of modification, in some instances. All this, in combination with global trade, has increased the probability of unintended, illegal and invisible presence of GMO derivatives in the food supply chain. Internet search engines, for example, can retrieve information about technological developments, laboratory and field trials, and the results of performance tests (Ruttink et al., 2010b). However, this type of information is often incomplete and sometimes even completely unavailable to the public. In the absence of accessible information about a specific GMO, this GMO will remain effectively unknown.

Cultural and regional differences in suitability, need and acceptance of GMOs is a potential cause of disputes. This is often reflected in regulations and their implementation. Within most jurisdictions, no import, use, or release of GMO-derived material is legal without prior authorisation. Requirements that must be met prior to GMO authorisation in the European Union (EU) include the availability of a validated and specific quantitative detection method and corresponding reference material (European Commission, 2003a). GMOs meeting these requirements, even those that are not authorised, can be classified as known GMOs. Notably, the distinction between known and unknown GMOs has to do with its characteristics rather than knowledge about the presence/absence of GMO in a particular product. There are a range of knowledge levels lying between the unknown and known GMOs, and these can be referred to as partially known or insufficiently known.

20.1.1 The novel characteristics of GMOs

Theoretically, developers of GMOs have an almost unlimited selection of genes (coding sequences) that they can introduce. However, the expression of the novel genes (transgenes) must be regulated in the recipient organisms, and this is done with promoters and terminators. The selection of functionally reliable promoters and terminators is much more limited than the selection of genes.

Genetically Modified and Non-Genetically Modified Food Supply Chains: Co-Existence and Traceability, First Edition. Edited by Yves Bertheau.

Transformation technology has also constrained the diversity of modifications that have been introduced. Consequently, GMOs commonly share at least some genetic marker sequences associated with the genetic modification. These shared markers can be targeted with screening methods. GMOs that can be detected using such screening methods are at least partially known. While most of the currently available and published GMO detection methods target known or partially known GMOs, this chapter will focus particularly on the detection of insufficiently known and unknown GMOs.

To better understand the limitations of analytical methods, it is necessary to briefly review the state-of-the-art methodologies for GMO detection in general. Before going into detail on detection methods, however, it may also be useful to consider the legal and knowledge-based status of GMOs in more detail. Among other things, this may clarify resource priorities and detection strategies.

20.1.2 Sources of unauthorised GMOs

Several incidents of illegal introduction of GMOs on the EU market have been reported over the last decade (see e.g. the European Rapid Alert System for Food and Feed; RASFF). The majority of cases concerned GMOs authorised and commercialised outside the EU, for example in the USA. Considerable documentation and often also reference materials and specific detection methods are available for these GMOs. However, a few cases concerned GMOs that had not been authorised anywhere, and where reference materials and specific detection methods were not available. Common to all reported incidents of unauthorised GMOs found in the food supply chain and released into the environment, is that they were all detectable with various screening methods. In other words, no incidents involving GMOs that were truly unknown *a priori* have yet been reported. Whether this is because no such GMO has been released into the environment or food chain, or whether it is just a reflection of the efforts and ability to detect unknown GMOs is, however, not evident.

Field trials are part of the performance assessment of GMOs, but they may lead to low level contamination of neighbouring fields. Birds or rodents may spread grains or seeds and incomplete sanitation or other human error may lead to unintended spread of viable material or unauthorised products into the food chain. Finally, intended distribution into the environment or food/feed chain cannot be completely ruled out. Ruttink et al. (2010b) outlined of an example of this with a case study leading to the detection of an illegally marketed product in the EU (Coban, i.e. tablets containing recombinant human intrinsic factor (rhIF) collected from dried, powdered transgenic *Arabidopsis* leaves and vitamin B12). To give another example: imagine a scenario in which a field trial is situated in a region where the local farmers are not well informed about the study or GMO issues. Here a local farmer may observe that plants in an experimental field (the field trial) are given special attention. For the local farmer, the plants in the field trial could be perceived as precious and attractive, tempting the farmer to try to obtain plant or seed samples from the field trial for his own personal use. The issue of GMOs originating from emerging countries is of growing importance as observed and reported via the RASFF in recent years.

20.2 CLASSIFICATIONS OF GMOs RELEVANT TO DETECTION

20.2.1 Legal classification of GMOs

The simplest distinctions between GMOs are binary, for example between legal and illegal, authorised vs. unauthorised, or deregulated vs. regulated. However, in many jurisdictions, these distinctions are not so simple. The legal domain of the application of a GMO may, for example, be limited to processing for food and/or feed, but not include planting or sowing. In this circumstance, a grain from a shipment that accidentally ends up in a field, germinates, and mixes with an intentionally planted crop is then illegal.

The GM maize event CBH351 (StarLink) was authorised for use in feed and industry but not in food by the USDA/EPA in the USA. When reported in food products in 2000, it caused serious concern for many stakeholders because it appeared outside the authorised domain of application and thus was illegal (Fox, 2001). Furthermore, the novel protein Cry9C in StarLink maize was believed to be a potential health risk for some humans.

A GMO can be tolerated (e.g. for a limited period of time and/or at concentrations below a defined threshold) or an authorisation can be withdrawn. If the product was already on the market, it is not always evident whether the now illegal GMO must be withdrawn immediately or if it is sufficient to stop further production and/or import. Indeed, the clearance duration of withdrawn GMOs in supply chains can be long. For instance, Starlink maize was detected in 2006 in shipments, five years after its official withdrawal (see http://www.epa.gov/pesticides/biopesticides/pips/starlink_corn_monitoring.htm; http://www.regulations.gov/search/Regs/contentStreamer?objectId=0900006480509565&disposition=attachment&contentType=pdf; http://www.twnside.org.sg/title2/health.info/twninfohealth057.htm).

The combination of transgenic traits into gene-stacked GMOs introduces an additional scenario. Applications for authorisation of two stacked maize GMOs from Syngenta are (in 2010) being evaluated in the EU. Both of these stacked GMOs are hybrids that include the maize event MIR162 as one of the parental events. However, MIR162 maize is not authorised in the EU as a single event, and no application for authorisation has been submitted. Thus, if the hybrids are authorised and MIR162 then appears alone in material on the EU market, the presence of MIR162 is, by definition, unauthorised. This is not an unlikely situation since the traits in hybrids are unlinked and therefore may segregate in pollen produced by the stacked hybrid plants. Such an authorisation of stacked GMOs without prior authorisation of each constitutive single GMO is also a matter of legal interpretations of European directives and regulations, a topic outside the scope of this chapter. Moreover, based on the already assessed stacked GMOs and the stacked GMOs in the authorisation pipeline, it appears that this strategy of requesting authorisation of stacked GMOs without prior authorisation of the single events is a growing new trend among notifiers.

Competent authorities may also consider available information related to risk assessments and traceability, for example. One of the major challenges facing global agricultural trade is the relatively common, low level presence (LLP) of GMOs authorised within the exporting jurisdiction but not (yet) authorised within the importing jurisdiction, an issue currently under discussion at the *Codex Alimentarius* level. This problem is particularly common for products originating from the USA and exported to the EU, and particularly with maize and soybean GMOs (events) for which applications for authorisation within the EU are being processed. It is commonly argued that such LLP should be tolerated, at least if the European Food Safety Authority (EFSA) expert panel on GMOs has conducted a risk assessment with a favourable conclusion for the GMO event involved. Notably, some third countries already approve the presence of unauthorised GMOs in imported feedstuff below a certain threshold (see Taverniers et al., Chapter 16 this book).

Analytical detection and discrimination of authorised and unauthorised GMOs may require slightly different tools and strategies, and thus also slightly different resources and priorities. However, the main paradigm shift with respect to analytical methods is associated with the distinction between known and unknown GMOs. Known GMOs can be detected with targeted methods. These are methods where the target is known *a priori*. For unknown GMOs the target is unknown in advance, by definition, and can only be described in detail *a posteriori*.

20.2.2 Knowledge based classification of GMOs

GM microorganisms (GMMOs) are almost exclusively produced and grown under contained conditions. This means that they are unlikely to escape. Furthermore, they have relatively small genomes (typically 3 to 50×10^6 base-pairs (Mbp)). This means that detection may be simpler than for GMOs with larger genomes. The present chapter will not deal further with GMMOs. There are still very few GM animals (GMAs) in commercial production, and these are limited to pets and ornamental fishes, although GMAs have also been developed for industrial production of drugs, particular proteins and food. Because of the limited relevance to the present day situation, this chapter will not deal further with GMAs, despite the fact that US authorities are currently considering the deregulation of GM salmon from AquaBounty. The vast majority of GMOs in commercial production and/or with the greatest potential of being introduced in the food supply chain are GMPs. Consequently, GMPs will be the focus of the rest of this chapter.

GMPs have genomes ranging in size from 120 Mbp to 20×10^9 base-pairs (Gbp), and transformation of GMPs typically involves the insertion of 2000 to 20 000 bp, or between 0.00001and 0.01% of the total size of the GMP genome. Of course, this means that detection requires extremely sensitive, and preferably targeted, analytical methods. As mentioned above, the selection of genetic elements that can be combined to functional gene cassettes is still relatively limited in reality, although in theory the selection is almost unlimited. The selection is, however, rapidly expanding as still more genomes are sequenced and genes characterised and functionally studied. Figure 20.1 schematically describes the transformation process of GMP, visualising sequence motifs that are particularly important for DNA-based detection methods.

Knowledge about the genetic elements that may be introduced, including the possible range of alternative codons, the actual use of the elements in commercialised GMPs and in field trials, and the origin and natural occurrence of the elements is extremely useful to the analysts and method developers. Such knowledge can be obtained from the biotechnology industry, for example as part of the documentation submitted with applications for authorisation of the GM products. It can also come from publicly available sequence databases, scientific literature and so on. The accessibility of this knowledge/documentation may, however, be limited for reasons like confidentiality

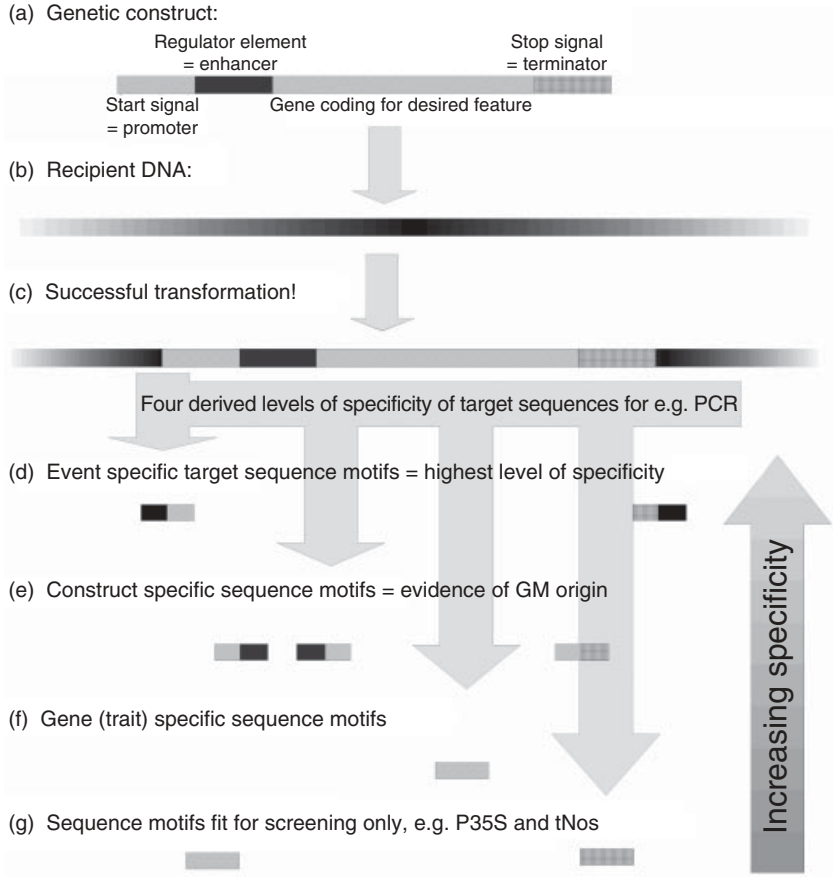

Figure 20.1. Elements involved in plant transformation to produce a GMO. A functional genetic construct is made by combining elements from different sources and this is then inserted into a recipient plant genome. A broad range of sequence motifs created during this process can be targeted for detection of the GMO. These targets can be grouped into four classes (in increasing order of specificity): screening motifs, gene specific motifs, construct specific motifs and event specific motifs. This classification of targets is widely adopted, including current ISO standards (ISO, 2005a, 2005b, 2006).

requirements to protect intellectual property rights, subscription requirements, etc. GMOs can be divided into at least four classes based on the availability of knowledge:

- **Knowledge class 1 (fully characterised GMOs)** A GMO of this class is fully characterised with respect to introduced and other affected DNA sequences, transcription regulation and production of novel proteins and so on. Event-specific detection methods are normally available and can easily be developed, and reference material is usually accessible or can be produced through synthesis of relevant DNA sequence fragments. Detection is consequently not a problem. All GMOs authorised in the EU under articles 7 and 19 of Regulation EC 1829/2003 (European Commission, 2003a) fall into this class of GMOs.
- **Knowledge class 2 (GMOs transformed with the same genetic construct(s) as GMOs in knowledge class 1)** The same genetic construct (or combination of neighbouring elements in the construct) can be used in the transformation of several different GMOs. In some cases, it may be necessary to distinguish such GMOs analytically, for example if one is authorised and the other not. Detection of the shared sequence motif of the

two or more GMOs may be logical as part of a fast and cost-efficient screening approach, but identification can require additional analyses, for example using event-specific methods, possibly in combination with quantitative measurements.

Examples include, but are not limited to, MON 809 and MON 810 maize both of which were transformed with the same plasmids (PV-ZMBK07 and PV-ZMGT10), T14 and T25 maize both of which were transformed with the same construct (P35S – *pat* – T35S) and MS1 x RF1 and MS1 x RF2 rapeseed both of which were transformed with the same constructs (PssuAra – *bar* – Tg7, PpTa29 – *barnase* – nos, PpTa29 – *barstar* – nos, Pnos – *nptII* – Tos) (CERA, 2010).

Gene-stacked GMOs are a special case. If a gene-stacked GMO is not a member of knowledge class 1, it may fall into knowledge class 2 if a parental GMO event is a member of knowledge class 1, that is the stacked GMO is either a hybrid between at least one member of knowledge class 1 and (an)other GMO(s) or (a) retransformed member(s) of knowledge class 1. For a more comprehensive discussion on gene stacking see Taverniers et al. (2008).

- **Knowledge class 3 (GMOs transformed with new combinations of genetic elements that include at least one element also found in one or more GMOs in knowledge class 1)** As mentioned above, the same genetic element may be used in more than one GMO, for example as a promoter, while at the same time the specific combination of elements is unique for each individual GMO. Typical examples are the P35S promoters (from cauliflower mosaic virus, CaMV and figwort mosaic virus, FMV), the T35S and Tnos terminators (from CaMV and the *Agrobacterium tumefaciens* nopaline synthase gene, respectively), *cp4-epsps*, *bar*, *pat*, *cry1A(b)* genes (encoding various novel proteins), and *nptII* and *bla* genes (marker genes for selection of transgenic isolates during the breeding process, derived from the cloning vectors in which the genetic construct is developed prior to transformation of the GMP).

  Screening methods for the detection of single genetic elements present in several GMOs may be very useful as a means of cost-efficient and rapid discrimination between samples with and without GMOs, and to reduce the number of candidate GMOs for which further identification analysis may be required in GMO-positive samples. However, the elements are almost invariably derived from natural sources such as bacteria, viruses or plants. Care is therefore required to discriminate between presence of the target due to presence of the natural source and due to presence of a GMO.
- **Knowledge class 4 (GMOs transformed with genetic elements that have not been used in the transformation of other GMOs, that is only novel elements)** As the selection of available and suitable genetic elements that can be used and combined to obtain the desired result of transformation increases, the probability that novel elements will be used also increases. Such novel elements are elements that have not been used in any other GMO, and where the available and/or accessible information may be very limited. This of course will make detection of the GMO much more challenging, since no targeted detection method will be available.

  It should be noted that even elements that theoretically fall in knowledge class 3, such as the CaMV P35S, can effectively belong to knowledge class 4 if the DNA sequence is modified in a way that will prevent the targeted detection methods from producing a positive signal (e.g. by introducing substitutions in the primer sites for the polymerase chain reaction; PCR).

20.3 DETECTION OF GMOs – A SHORT REVIEW

The targets of GMO detection methods are either an element of the genetic modification itself (i.e. a DNA sequence motif) or a derived novel product such as a protein. The target may be common to several GMOs or unique to a single GMO. In the latter case, it is necessary to discriminate between a target that is by nature unique and one that may be unique at a particular moment but could later be introduced to additional GMOs. The technologies used to detect DNA sequence motifs and proteins differ, but the principles of results interpretation, for additional verification and so on are largely the same. However, the DNA sequence is present in all developmental stages of the organism, while the protein may be present in only a limited developmental phase and/or its presence may depend on the genetic background (Stave, 2002). In the following section we will therefore focus on these principles, using DNA-based technologies to exemplify the processes. Technology-specific aspects will only be discussed where a clear distinction between technologies is required to understand these principles. The only truly unique GMO-specific targets are the event-specific integration border DNA sequence motifs stemming from the fusion of an introduced sequence and the insertion locus of the receiving genome (see Figure 20.1).

Traceability facilitates identification of the origin of material. Global information networks, databases, and so

on may provide information about developments of new GMOs, novel genetic elements that are potentially exploitable and authorisations outside the stakeholder's own jurisdiction. This type of information can be used by stakeholders to improve their ability to detect, identify and characterise unauthorised or unknown GMOs, as well as to prioritise developments and applications of particular analytical methods (Ruttink et al., 2010b).

Development of analytical methods and strategies for detection, identification and characterisation of unauthorised and unknown GMOs has been a major priority within the Co-Extra project. In parallel, a modular decision support system (DSS) has been developed in which traceability and other information can also be taken into consideration (see Bohanec et al., Chapter 25 in this book). Other related initiatives have been described elsewhere, for example the *GMOtrack* (Novak et al., 2010; see also Ruttink et al., 2010b). These developments together may be exploited by the analytical laboratories and other stakeholders to significantly reduce the challenges posed by unauthorised and unknown GMOs.

Ruttink et al. (2010b) distinguished between an analyte-centred and a product-centred approach. The former is typically applied to routine analyses and is described in the following paragraphs. The product-centred approach is more complicated and starts with an analysis of available information, and leads to the design of an *ad hoc* sampling and detection strategy.

The starting point for the analytical laboratory, unless very specific information is available on the sample, should typically be to determine the species composition and degree of processing of ingredients in the sample. Even presumed single ingredient and unprocessed samples may prove to contain enough unexpected material belonging to (an)other species to confuse the analysis and results interpretation. Only the use of event-specific methods can lead to immediate and correct identification of unexpected material. Processing in particular, but also the effect of diluting ingredients by mixing with other ingredients, may reduce the extractability or the concentration of the target relative to the total mass of DNA sequences or proteins. This will affect the detectability and quantifiability of the target negatively; namely the sample-specific limit of detection (LOD) and limit of quantitation (LOQ) will increase (Berdal and Holst-Jensen, 2001; Holst-Jensen et al., 2003).

Determination of species composition can be easily achieved using screening methods. Multiplex methods, meaning methods capable of simultaneous detection and identification of multiple targets, may be more cost effective than series of individual analyses for each species. Several methods with degrees of multiplexed species identification have been developed and published, and some of these will be discussed further in this chapter. However, available endogenous reference tests for particular taxa, such as sugar beet and potato, are not sufficiently specific although the quantitative method has been accepted by the EURL-GMFF (QPCRGMOFOOD, 2004; unpublished data).

The analytical laboratory will almost invariantly have to test for the presence of more than one GMO in the sample. Screening methods may again prove very useful for this purpose. As explained in the section on knowledge-based classification above, various analytical targets can be shared by many or a few GMOs, while others can be more or less unique to a single GMO. Furthermore, each target can be combined with other targets in a combination shared by only a few GMOs or even unique to a single GMO. This can be exploited to rationalise the GMO detection process by implementing what is often referred to as the 'Matrix Approach' (see e.g. Querci *et al*., (2010) and was first outlined in 2001 in the workplan of the European GMOchips research project, G6RD-CT2000-00419, see http://www.bats.ch/gmochips/introduction/). For each GMO, the response to specific detection methods (modules) can be tabulated (Figure 20.2). Then, if the sample is analysed using these modules, the observed response pattern of the sample can be compared to the tabulated data, and the set of GMOs that might be present can be rapidly narrowed, often to none, a single or only a few GMOs. Confirmatory analyses using event specific methods (modules), for example, may be required. However, there is obvious cost efficiency to performing up to three confirmatory analyses rather than up to 20, or even more, event-specific analyses for each sample.

Multiplex methods for simultaneous detection and identification of particular species and GM specific/derived targets are already published and one is also commercialised (Chaouachi et al., 2007; Hamels et al., 2009; Rønning et al., 2005). A single method could potentially allow the analytical laboratory to detect and identify all GMOs in each sample. However, such a method still might not be reliable. For example, if the sample contains very different concentrations of different targets, then the least abundant target may not be detectable because the signal produced by that target is obscured by the much stronger signal produced by the dominant target(s). As a matter of fact, establishing a scheme for formal validation of multiplex methods (see Bellochi et al., Chapter 21 this book) was one of the most important achievements of the Co-Extra

Species	GMO	Auth. status	Screen A	Screen B	Screen C	Screen D	Screen E
Cotton	Cotton A	A	+	-	-	-	-
	Cotton B	A	-	+	-	-	-
	Cotton C	U	+	+	-	-	-
Maize	Maize A	A	+	-	-	-	-
	Maize B	A	+	+	-	-	-
	Maize C	A	+	-	+	-	-
	Maize D	A	+	-	+	+	-
	Maize E	A	-	+	-	-	+
	Maize F	A	+	-	-	+	-
	Maize G	U	+	-	-	+	-
	Maize H	U	+	+	-	-	-
	Maize I	U	+	-	-	+	+
Potato	Potato A	A	+	+	-	-	-
	Potato B	A	+	+	-	+	-
	Potato C	U	+	+	+	-	-
Rapeseed	Rapeseed A	A	+	-	+	+	+
	Rapeseed B	A	+	+	-	-	-
	Rapeseed C	A	+	+	-	+	-
	Rapeseed D	U	+	+	+	-	-
	Rapeseed E	U	-	+	-	+	-
Rice	Rice A	U	+	-	-	+	-
	Rice B	U	-	+	-	+	-
	Rice C	U	+	-	+	-	+
	Rice D	U	+	+	-	-	-
Soybean	Soybean A	A	+	+	-	+	-
	Soybean B	A	-	+	-	+	-
	Soybean C	A	-	-	+	-	+
	Soybean D	U	+	-	-	-	+
	Soybean E	U	-	+	-	+	-
	Soybean F	U	+	-	+	-	-
Sugar beet	Sugar beet A	A	+	+	-	-	-
	Sugar beet B	U	+	-	+	-	-

Figure 20.2. Screening table. A number of GMOs are included for each species, some of which are authorised (indicated by A in light grey cell) while others are unauthorised (indicated by U in grey cell). Five screening modules, specific to a particular target sequence associated with genetic modifications are used. For each screening module, the result of application of the module to material for each individual GMO is indicated as positive (+) or negative (–).

project (www.coextra.eu), and is particularly relevant for ensuring reliable detection and identification of all GMOs, including those that are not authorised.

In the following two sections, we present two examples to illustrate the process and application of the principles of the matrix approach. Examples of the matrix approach, with the associated analytical methods that are routinely applied in GMO testing laboratories, are described in several other publications (e.g. Hamels et al., 2009; Van den Bulcke et al., 2010 and Waiblinger et al. 2008; 2010). Decision support systems such as the *GMOtrack* (Novak et al. 2010) or the Co-Extra DSS (see Bohanec et al.,

Chapter 25 this book) may facilitate optimal implementation of the matrix approach.

20.3.1 Example case 1 – maize flour: a single low processed ingredient

The laboratory receives a sample, described as maize flour, imported from the USA. The material is presumably low processed, thus the amount and integrity of DNA and protein is expected to be high. It cannot be excluded that the maize is contaminated with minor quantities of material from other species, but it is reasonable to assume that more than 95% of the sample is maize-derived.

The laboratory may apply protein-based testing methods. Typically this allows for discrimination between various Cry proteins, various EPSPS proteins and so on, but not for discrimination between GMOs that produce the same form of the same protein (Van den Bulcke et al., 2007). If the laboratory applies DNA-based testing methods, it may be possible to detect and discriminate groups of related GMOs as well as single events. In the following example, a DNA-based approach is described. The choice of DNA extraction method could affect the result, but the laboratory selects a method that has repeatedly been demonstrated to produce high yields of DNA without detectable inhibitors. The Co-Extra DSS (Bohanec et al., Chapter 25 this book) can be used by the analytical laboratory to select appropriate DNA extraction modules. An approach for validation of DNA extraction modules for particular materials was also developed within Co-Extra (see Bellochi et al., Chapter 21 this book).

First, the laboratory screens for presence of soybean and rapeseed, as well as for five common promoter, terminator or fusion sequence motifs. Then, based on the observed presence/absence pattern for these screens, a list is produced naming the GMOs that might be present in the sample. This list may include both authorised and unauthorised GMOs (Figure 20.3). The tests are performed using quantitative modules, so the results may already indicate whether the sample complies with the EU legal threshold for labelling (European Commission, 2003b).

Next, the laboratory chooses to apply specific tests for presence of the listed unauthorised GMOs, but suitable detection modules are only available for some of the unauthorised GMOs. In this case, it is assumed that qualitative tests are sufficient (Figure 20.3).

20.3.2 Example case 2 – poultry feed: a processed and mixed sample

The laboratory receives a sample consisting of pellets, described as poultry feed, with a vague description of the ingredients. Apparently the feed is produced in Europe, but the ingredients may come from sources outside Europe. The material contains at least some ingredients that are presumably highly processed, thus the amount and integrity of DNA and protein is uncertain. Since protein-based methods generally have inferior LOD compared to PCR, the laboratory decides to use PCR for the analysis. Furthermore, some of the ingredients may compromise PCR analyses, so the choice of the DNA extraction module is critical. The laboratory chooses a fairly complex DNA extraction protocol, including several steps to remove PCR inhibitors and increase the purity of the DNA.

First, the laboratory screens for presence of maize, soybean, potato, rapeseed, cotton, sugar beet and rice, as well as for the same five common promoter, terminator and fusion sequence motifs as in example case 1. Again, based on the observed presence/absence pattern for these screens, a list is produced that names the potential GMOs that could be present in the sample (Figure 20.4). Because of the presence of very unequal quantities of ingredients from several of the species, it is not possible to determine if the sample is in compliance with the labelling threshold at this stage.

Next, the laboratory chooses to perform quantitative tests for three particular GMOs, based on the assumption that the observed results of the screening can be explained if the quantity of one of these three events exceeds the labelling threshold (Figure 20.4).

The quantitative results in this example do not indicate non-compliance with the labelling threshold, so finally the laboratory considers the need to test for the presence of unauthorised GMOs. In this case, they choose to perform analyses (qualitative) for only five of the unauthorised events on the original list, because these belong to the dominant ingredients, maize and soybean. For the other ingredients, it is assumed that the presence of any unauthorised GMO is unlikely to be detected because such presence is almost invariantly at the trace level and therefore well below the sample-specific LOD (Figure 20.4).

20.3.3 Multiplex GMO detection methods – examples

Several multiplex GMO detection methods were developed within the Co-Extra project (see Pla et al., Chapter 19 this book). The majority were based on the PCR technique at some stage, but adaptations were either introduced or alternatives to PCR were alternatively introduced to reduce some of the most problematic aspects of PCR. These problems include:

Species	GMO	Auth. status	Screen A	Screen B	Screen C	Screen D	Screen E	
Cotton		Not tested						
Maize	Maize A	A	+	-	-	-	-	**Possible** **< 0.9%**
	Maize B	A	+	+	-	-	-	**Possible** **< 0.9%**
	Maize C	A	+	-	+	-	-	Not possible
	Maize D	A	+	-	+	+	-	Not possible
	Maize E	A	-	+	-	-	+	Not possible
	Maize F	A	+	-	-	+	-	Not possible
	Maize G	U	+	-	-	+	-	Not possible
	Maize H	U	+	+	-	-	-	**Possible** **Event specific test**
	Maize I	U	+	-	-	+	+	Not possible
Potato		Not tested						
Rapeseed		**Negative in test**						
Rice		Not tested						
Soybean	Soybean A	A	+	+	-	+	-	Not possible
	Soybean B	A	-	+	-	+	-	Not possible
	Soybean C	A	-	-	+	-	+	Not possible
	Soybean D	U	+	-	-	-	+	Not possible
	Soybean E	U	-	+	-	+	-	Not possible
	Soybean F	U	+	-	-	-	-	Not possible
Sugarbeet		Not tested						
SAMPLE	**Test result**		Positive 0.5% of maize	Positive 0.3% of maize	Negative	Negative	Negative	Negative

Figure 20.3. Example of analysis of single ingredient sample. The declared ingredient is maize, but the laboratory chose to also test for possible presence of impurities from rapeseed and soybean. Rapeseed is not detected, but soybean is. However, the results of the screening tests indicate that for all the soybean GMOs and for six of the maize GMOs in the screening table at least one target is not detected (indicated by white + in dark grey cell) and consequently (angled arrow lower right) their presence is 'not possible'. For each of these GMOs the laboratory therefore concludes that the sample is negative at the limit of detection. For three maize GMOs in the screening table, all targets are detected. Two of these GMOs are authorised, and since the quantity of the screening targets is clearly below 0.9% it is concluded that if the positive screening results are due to presence of these GMOs, labelling is not required. The third GMO that might be present, however, is not authorised. Therefore, the laboratory chose to perform a qualitative event-specific test for this GMO. In this case, the event-specific test result is negative, and the laboratory therefore concludes that the sample most likely contains only authorised maize GMOs at a concentration well below 0.9%.

Species	GMO	Auth. status	Screen A	Screen B	Screen C	Screen D	Screen E	QN test ?	Event specific test?
Cotton		**Negative in test**							
Maize	Maize A	A	+	-	-	-	-	**Possible**	**Yes! <0.3%**
	Maize B	A	+	+	-	-	-	**Possible**	**Yes! <0.3%**
	Maize C	A	+	-	+	-	-	Not possible	
	Maize D	A	+	-	+	+	-	Not possible	
	Maize E	A	-	+	-	-	+	**Possible**	**No**
	Maize F	A	+	-	-	+	-	**Possible**	**No**
	Maize G	U	+	-	-	+	-	**Possible**	Yes! Negative
	Maize H	U	+	+	-	-	-	**Possible**	Yes! Negative
	Maize I	U	+	-	-	+	+	**Possible**	**Yes! Positive**
Potato		**Negative in test**							
Rapeseed	Rapeseed A	A	+	-	+	+	+	Not possible	
	Rapeseed B	A	+	+	-	-	-	**Possible**	**No**
	Rapeseed C	A	+	+	-	+	-	**Possible**	**No**
	Rapeseed D	U	+	+	+	-	-	Not possible	
	Rapeseed E	U	-	+	-	+	-	**Possible**	**No**
Rice		**Negative in test**							
Soybean	Soybean A	A	+	+	-	+	-	**Possible**	**Yes! <0.2%**
	Soybean B	A	-	+	-	+	-	**Possible**	**No**
	Soybean C	A	-	-	+	-	+	Not possible	
	Soybean D	U	+	-	-	-	+	**Possible**	Yes! Negative
	Soybean E	U	-	+	-	+	-	**Possible**	Yes! Negative
	Soybean F	U	+	-	+	-	-	Not possible	
Sugar beet	Sugar beet A	A	+	+	-	-	-	**Possible**	**No**
	Sugar beet B	U	+	-	+	-	-	Not possible	
SAMPLE	**Test result**		Positive	Positive	Negative	Positive	Positive		

Ingredient quantities: maize (very high) > soybean (medium) > rapeseed (low) > sugarbeet (very low)

Figure 20.4. Example of analysis of a complex sample with mixed ingredients and processed material. The laboratory has no reliable information *a priori* about the ingredients and therefore chose to perform quantitative tests for all seven species listed in the screening table. Cotton, potato and rice are not detected, while the quantity of the other four species vary from very high to very low (see bottom of figure). The target of screen C should be present for two of the maize GMOs, two of the rapeseed GMOs, two of the soybean GMOs and one of the sugarbeet GMOs in the screening table. Since this target is not detected, the laboratory concludes that none of these seven GMOs is present (at the limit of detection). For the remaining nine authorised and six unauthorised GMOs listed in the screening table all targets were detected (angled arrow lower right). The ingredient quantities for rapeseed and sugarbeet are too low to justify testing for the four GMOs belonging to these species, because the limit of detection and/or quantification would be unsatisfactory (see circles and arrows at bottom). Among the maize and soybean GMOs authorised and possibly present, two maize and one soybean have dominating market shares and their presence may explain the observed quantities of the screening targets. Consequently, the laboratory chose to perform quantitative (QN) tests for these three GMOs. Quantitative tests for the two other authorised maize and the second authorised soybean GMO are not performed by the laboratory because it is considered that these three GMOs have low market shares and are less likely to be present. The laboratory also needs to confirm that no unauthorised GMO is present in the sample, and therefore event-specific tests are performed for the three unauthorised maize and two unauthorised soybean GMOs that are possibly present. In this case, the quantity of authorised GMOs indicate that the sample does not require labelling. However, the event-specific tests for the unauthorised GMOs indicate that a specific unauthorised GMO (maize I) is present.

- high sensitivity to impurities in template DNA, resulting in partial or complete inhibition of the amplification process;
- competitive effects that lead to bias favouring targets that are either predominant in the template DNA prior to PCR or targets that for one reason or another amplify more efficiently than others;
- possible unspecific amplification or amplification of chimeric targets.

Pooling of products from PCRs, followed by array hybridisation to identify the amplified targets, is a fairly simple approach that has been applied to GMO detection several times, both within and outside the Co-Extra project (Hamels et al., 2009; Leimanis et al., 2006; Rønning et al., 2005; Xu et al., 2005). By performing the PCRs as oligoplex reactions (typically between two and six primer pairs simultaneously) this approach may prove to be cost efficient, in particular if the samples analysed do not contain more than a few species and/or GMOs. This is exemplified with the successful collaborative trial validation of such an assay in the Co-Extra project (Leimanis et al., 2008).

A related approach, that involves substituting the array-based detection step with capillary gel electrophoresis (CGE), thereby discriminating the products by a combination of size and label colour (SC-CGE), was also developed in the Co-Extra project (Nadal et al., 2006, 2009) and was also successfully validated with a collaborative trial (see Bellocchi et al., Chapter 21 this book). Heide et al. (2008b) developed a multiplex qualitative PCR assay using PCR primers that incorporated a common 5′-universal tail motif. This method is reported to result in more equal amplification rates for all targets. Detection and identification of the amplified targets was done using SC-CGE. Heide et al. (2008a) later modified their approach to make it quantitative by introducing a two-step PCR. The first step involved the incorporation of the universal 5′-tail followed by trypsin digestion of the DNA polymerase. The second step involved PCR with the universal tail primers only. This approach was developed even further, into a ligation-mediated PCR assay (Holck et al., 2009) in which the universal tail primer motif was first introduced to each target-template by ligation of two probes, each corresponding to one half of the target-template with the universal tail at its descending end.

Chaouachi et al. (2008) used a similar approach to Holck et al. (2009) to develop a single nucleotide polymorphism genotyping (SNPlex) based assay. However, for each target one of the ligation probes contained a unique zip-code label that was used in the detection/identification step. The SNPlex assay was demonstrated to perform well with as many as 48 targets, including targets for multiple species, screening markers and GMOs. Prins et al. (2008) also used ligation and zip-codes, but their approach was

to develop circular probes upon ligation. Probes could only be amplified by PCR if they were circularised. Detection was done on arrays using the zip-codes as capture probes.

Morisset et al. (2008) used a completely different amplification approach called NASBA, in which RNA is amplified instead of DNA. Coupled with array analysis, this approach (NAIMA) appears to be less prone to several of the drawbacks of multiplexing PCR, and seems to provide quantitative or semi-quantitative results. Thus, NAIMA is a candidate to challenge PCR when multiplexing and/or quantitation is needed. However, the performance of the NAIMA approach with high levels of multiplexing must still be demonstrated.

Outside the Co-Extra project, three particularly interesting approaches for multiplexing have been explored. The first is the use of the Luminex X-map technology for multiplex target identification and quantitation (Fantozzi et al., 2008). The second is the use of microtiter plates prespotted with primers and probes for real-time PCR (Mano et al., 2009; Querci et al., 2009). The third is the use of DNA fingerprinting techniques to produce construct or event-specific profiles, simultaneously facilitating the detection of knowledge class 3 GMOs and sequencing-based confirmation/identification (Raymond et al., 2010; Ruttink et al., 2010a).

20.4 DETECTION OF UNAUTHORISED GMOs

Detection of unauthorised GMOs is not necessarily very different from detection of authorised GMOs. The only real difference is associated with the degree of knowledge the analytical laboratory has, *a priori*. Thus, the knowledge-based classification described in Section 20.2.2 is directly linked to detectability.

20.4.1 Qualitative detection of unauthorised GMOs

For a GMO belonging to knowledge class 1 and 2, and possibly also to class 3, detection can be made with the same tools and analytical strategies independent of the legal status of the GMO (authorised or not). By definition, authorised GMOs never belong to knowledge classes 3 and 4 and detection of GMOs in these knowledge classes can be quite challenging. For detection of a GMO in knowledge class 3 with a module targeting a common DNA sequence motif, it may be necessary to exclude the possibility that the positive signal is caused by an authorised GMO, an unauthorised GMO belonging to knowledge class 1 or 2, and any non-GM source such as a soil bacterium or a virus. This can be achieved with a broad set of controls, but it does not provide a description of the GM source itself. Thus, it may be necessary to use cloning and sequencing to obtain the information needed to allow the analytical laboratory or others to conclude that an unauthorised GMO is present with the required certainty.

Ruttink et al. (2010a) reviewed some of these issues and proposed the use of a DNA-fingerprinting approach. A related approach has also been proposed by Raymond et al. (2010). One of the advantages is that fingerprints can be tabulated and communicated between laboratories, provided that they can be reproduced in other laboratories, as demonstrated by Raymond et al. (2010). Another advantage is that the fragments produced from fingerprinting can be sequenced, thus facilitating identification and confirmation of the identity of the fragment. This could contribute to increasing the available knowledge for GMOs where, for example, little sequence information was available *a priori*. However, fingerprinting cannot be applied to GMOs belonging to knowledge class 4 without some prior analysis (see Section 20.5).

For a GMO in knowledge class 4 (unknown GMO), the analytical laboratory by definition has no *a priori* knowledge about either the newly introduced genetic elements or their associated products or features. In principle, anything could have been introduced, so the obvious thing would be to start looking for anything that is present in the suspected GMO but absent in the non-GM counterpart. Various -omics tools may be applied, but this will only lead to detection in exceptional circumstances unless the biochemical analyses are combined with well-planned bioinformatics strategies for data analysis. An important question, however, is whether a laboratory will ever receive a sample for knowledge class 4 analysis without at least some additional information to help the analytical laboratory to design a significantly more efficient strategy than a direct blind -omics based comparison of data produced with the suspected GMO and a non-GM counterpart. A DSS could then prove useful (see Bohanec et al., Chapter 25 this book) as could other approaches that exploit multiple sources of information treated systematically (Ruttink et al., 2010b).

For example, if the sample was taken based on a specific suspicion (e.g. observations of a particular environmental or health effect) then there is already some information implicating particular classes of genes or proteins. Alternatively, a particular product with exceptionally high yield or tolerance to a harsh environment might be reported and sampled. Again, this would implicate particular classes of genes and proteins. Finally, if the sample is taken because of intelligence, the intelligence report may also include details on the purpose of the release and/or development

of the suspected GMO and again this may point to particular classes of genes and proteins. The detection of unknown GMOs is further discussed in the next section (20.5).

20.4.2 Quantitative detection of unauthorised GMOs

With the matrix approach and qualitative analyses it is often impossible to determine if the observed pattern is produced by one, two or many GMOs. In these cases it is therefore also difficult to determine if the pattern is produced entirely by authorised GMOs or if one or more unauthorised GMOs are also involved, as exemplified in Figure 20.4. The use of fingerprinting and/or quantitative methods may provide the additional information required to conclude that unauthorised GMO is present in a sample.

Cankar et al. (2008) described a differential quantitative PCR (dQ-PCR) approach to detect unauthorised GMOs. They exemplified this with the common CaMV P35S. The sources of the CaMV P35S can be a broad spectrum of GMOs belonging to several different species, as well as the CaMV itself and plants descending from ancestors where CaMV sequences were introduced during their evolution. Thus, if the observed quantity of CaMV P35S is statistically significantly higher than the sum of all authorised GMOs carrying the CaMV P35S plus all naturally derived CaMV P35S in the sample, then the additional CaMV P35S that is not explained by confirmed sources is reasoned to be derived from unauthorised GMOs.

The applicability of the differential quantitative PCR approach is not limited to the CaMV P35S. Even if only screening modules are used in combination with a matrix approach-based strategy, the quantitative data could be interpreted to implicate the likely presence of a particular unauthorised GMO, allowing the analytical laboratory to apply an event-specific module that directly targets this GMO. An inter-laboratory validation of the dQ-PCR method has recently been conducted (Ancel et al., manuscript in preparation).

20.5 DETECTION OF UNKNOWN GMOs

20.5.1 Application of genomics – DNA sequence based analysis

If the composition of the non-GM recipient genome is known at the sequence level, then a deviation from this could be of GM origin. More and more completely sequenced genomes are published, and this is expected to continue at an even greater rate in the coming years. Nesvold et al. (2005) proposed that microarrays be designed with millions of hybridisation probes that could be used to screen suspected GMP DNA for any novel sequence motif. Nesvold et al. (2005) also described how data could be processed to facilitate identification of novel genetic modifications. The approach was exemplified *in silico*, but has never been demonstrated *in vitro*, largely because the approach for array design would require a new microarray for each species, and the cost per set of arrays would (at present) be extremely high.

A simpler approach would be one in which the analytical laboratory starts with a compilation of thousands of genes, promoters and terminators that theoretically could be used successfully in the development of a GMO. These genes, promoters and terminators could be grouped according to function, likely type of application or assumed fitness for particular hosts. Then from this compilation, variants of the genes could be constructed *in silico*, for example taking preferred host codon usage into consideration. Similarly, for promoters and terminators the laboratory could consider ways that these genetic elements could be modified by a GMO developer. Finally, groups of compiled sequences could be subjected to probe design.

This approach would permit detection of some unknown GMOs but not of GMOs with entirely novel and unknown elements. Thus it can be seen as a compromise between pragmatism (targeting the more likely used promoters, genes, terminators, cloning vectors, etc.) and flexibility and coverage (unbiased screening for all possible genetic elements).

Tengs et al. (2007) applied such a strategy to detect introduced sequence motifs in two transgenic *Arabidopsis thaliana* and one transgenic rice line. These plant species both have small genomes, and attempts to apply the approach to plants with larger genomes were not successful. The array design applied by Tengs et al. was based on a compilation of all published cloning vectors containing the CaMV P35S, that is it included all genetic elements that had been present in at least one cloning vector that also contained the CaMV P35S. Notably, the transgenic rice line included in their study did not contain the CaMV P35S. Tengs et al. (2010) modified their approach to include additional targets, used larger probes and a two-colour labelling system that also allowed them to apply the microarrays to species with considerably larger genomes (soybean and maize).

The sequence motifs identified by array analysis suffice to continue with PCR (fingerprinting) and DNA sequencing, thus allowing for a fairly detailed description of the GMO and its associated features. This would facilitate the first temporary risk assessment and risk management after detection of the unknown GMO.

With the progress made in DNA sequencing technology, it might even be feasible for smaller genomes and gradually for larger genomes to do a complete genome sequencing of a specimen. This would then permit either direct identification of the transgenic elements or a comparison between a non-GM reference genome and the genome of the suspected GMO, to indirectly identify any transgenic elements.

20.5.2 Application of transcriptomics – RNA transcript sequence analysis

The approach taken by Nesvold et al. (2005) can be taken one step further, based on the assumption that the purpose of the genetic modification is to introduce a novel gene that will be expressed by transcription (a novel mRNA is produced) and translation (a novel protein is produced). Comparing the transcriptome or the proteome of the suspected GMO with that of a non-GM individual may provide suitable evidence of the presence of a GMO and allow the analytical laboratory to provide a first description of the GMO and its features.

Tengs et al. (2009) performed a transcriptome-based study on food plants in which high-throughput sequencing of transcripts (cDNA) was followed by *in silico* subtraction of the cDNA library against published transcripts and genomic DNA, in order to identify the transcripts unique to the suspected GMO. Including several tissues and/or environmental samples in the analysis might be useful since the transcription of genes is partly tissue dependent and partly affected by environmental exposure. On the other hand this would obviously increase the total analytical costs. Tengs et al. also proposed that *in vitro* subtraction of cDNA libraries from the suspected GMO and a non-GM reference line of the same species could be used to increase the concentration of novel transcripts prior to high-throughput sequencing.

20.6 CONCLUSION

Technological progress has made it possible for almost anybody with a minimum of resources to create a GMO, see http://biohack.sourceforge.net/ and other references to bio-hacking. The limitations to development of GM plants or animals are constantly decreasing. Detection of unknown GMOs is unlikely to become routine in any GMO laboratory. Both the technology and workload required for sample analysis are limiting factors. Furthermore, taking samples for such analysis may require *ad hoc* sampling schemes, unlikely to be set up without *a priori* knowledge analysis (see also Ruttink et al., 2010b). However, ignoring the possible presence of unknown GMOs in the food supply-chain or elsewhere cannot be justified due to the possible consequences to society, health or the environment. International collaboration is required to ensure efficient monitoring and intelligence to identify efforts to release unauthorised GMOs in general and unknown GMOs in particular, as recently outlined by the US General Accountability Office (GAO, 2008). Analysis of samples may have to be done in specific laboratories that are typically equipped with a combination of highly advanced analytical equipment, databases with extensive sequence information including confidential parts of dossiers and patent applications and so on. So far, no GMO belonging to knowledge class 4 has ever been reported. Unless the necessary resources and efforts are provided to monitor for presence of such GMOs it is likely that the first detection of a knowledge class 4 GMO will occur after it causes harm and at least some of its negative consequences are already irreversible.

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