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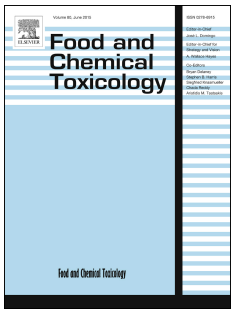

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PII: S0278-6915(17)30483-0

DOI: 10.1016/j.fct.2017.08.032

Reference: FCT 9249

To appear in: *Food and Chemical Toxicology*

Received Date: 17 March 2017

Revised Date: 30 June 2017

Accepted Date: 22 August 2017

Please cite this article as: Nadal, A., De Giacomo, M., Einspanier, R., Kleter, G., Kok, E., McFarland, S., Onori, R., Paris, A., Toldrà, Mò., van Dijk, J., Wal, J.-M., Pla, M., Exposure of livestock to GM feeds: Detectability and measurement, *Food and Chemical Toxicology* (2017), doi: 10.1016/j.fct.2017.08.032.

EXPOSURE OF LIVESTOCK TO *GM* FEEDS: DETECTABILITY AND MEASUREMENT

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ABSTRACT

This review explores the possibilities to determine livestock consumption of genetically modified (GM) feeds/ingredients including detection of genetically modified organism (GMO)-related DNA or proteins in animal samples, and the documentary system that is in place for GM feeds under EU legislation. The presence and level of GMO-related DNA and proteins can generally be readily measured in feeds, using established analytical methods such as polymerase chain reaction and immuno-assays, respectively. Various technical challenges remain, such as the simultaneous detection of multiple GMOs and the identification of unauthorized GMOs for which incomplete data on the inserted DNA may exist. Given that transfer of specific GMO-related DNA or protein from consumed feed to the animal had seldom been observed, this cannot serve as an indicator of the individual animal's prior exposure to GM feeds. To explore whether common practices, information exchange and the specific GM feed traceability system in the EU would allow to record GM feed consumption, the dairy chain in Catalonia, where GM maize is widely grown, was taken as an example. It was thus found that this system would neither enable determination of an animal's consumption of specific GM crops, nor would it allow for quantitation of the exposure.

KEYWORDS

Animal tissue; DNA and protein transfer; exposure assessment; genetically modified organism (GMO); livestock; traceability

RESEARCH HIGHLIGHTS

- Reviewed data show that both DNA- and protein-based methods can be used to detect genetically modified ingredients in feed
- Transfer of transgenic DNA/proteins from consumed feed to animal tissues is not a good biomarker for GM exposure measurement
- Traceability documentation for GM crops in the EU allow for identifying animals having consumed GM feed ingredients to a limited extent
- Traceability documentation is not able to quantify the intake and identify the specific genetically modified crops involved

ABBREVIATIONS

bp, base pairs; Bt, *Bacillus thuringiensis*; cp, chloroplast; CTAB, cetyltrimethylammonium bromide; ddPCR, droplet digital polymerase chain reaction; EFSA, European Food Safety Authority; ELISA, Enzyme-Linked Immunosorbent Assay; EU, European Union; GI, gastrointestinal; ISO, International Organization for Standardization; GM, genetically modified; GMO, genetically modified organism; LOD, limit of detection; NEP, newly expressed protein; NGS, next-generation sequencing; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; UGMO, unauthorized genetically modified organism

1. INTRODUCTION

Since the first large-scale commercial introduction of genetically modified (GM) crops two decades ago, there has been an almost steadily increasing adoption of such crops by farmers around the world. In 2015, the global area planted to these crops amounted to 180 million hectares, whilst countries growing more than a million hectares each were situated in North, Central and South America, China, the Indian subcontinent and South Africa. The largest part is made up of commodity crops, including soybean, maize, cotton and canola (ISAAA, 2016). Besides food applications, these crops are known to have feed applications as well, for example as protein-rich meals retained after extraction of vegetable oils from oilseeds such as soybean, rapeseed, and cottonseed, or starch from maize. Cultivation of GM crops within Europe has been limited in overall scale and confined to a few countries, particularly Spain (ISAAA, 2016), where farmers in several northern regions grow a particular version of insect-resistant maize. Notwithstanding this limited cultivation in Europe, GM crops and derived products can enter the European livestock feed supply chain through imports of feed commodities from GM-crop-growing countries given that a range of GM canola, cotton, maize and soybean events have been approved for import, processing, and food and feed use within the EU (EU, 2017).

Before a GM food or feed is placed onto the market, it has to undergo a regulatory approval procedure under EU legislation, a situation which is similar to many non-EU countries. Part of this EU-wide approval procedure is a rigorous pre-market assessment of the new crop's safety for human consumers and livestock animals. The outcomes of such an assessment will then form the basis of a decision for market approval. Whilst the regulations may differ from one country to another, the safety assessment of GM foods and feeds is commonly carried out according to an internationally harmonized approach, which has been laid down in guidelines of the Codex Alimentarius commission (Codex alimentarius, 2008) for the safety assessment of foods from

plants modified with recombinant DNA technology. These guidelines are also the basis for the elaborate guidance developed by the European Food Safety Authority's Panel of experts on genetically modified organisms (EFSA GMO Panel) (EFSA, 2011a). With a few modifications, this guidance has been incorporated as an annex into a legislative piece, namely Implementing Regulation (EU) No 503/2013 (EU, 2013). Whilst the pre-market safety assessment is a prerequisite for regulatory approval, EU legislation offers the option for decision makers to require additional case-specific post-market monitoring of a given GM product as part of the decision to approve the latter's introduction into the market (EU, 2003a). Such monitoring could help to verify assumptions about intake of the product by consumers and livestock, and risk identified during the pre-market assessments. So far, monitoring has not been required for GM feeds specifically. It is only for several GM soybeans with modified oil composition that post-market data on actual import and consumption of refined seed oils have to be collected for the purpose of verifying pre-market consumption estimates made for the assessment of potential impact on human nutrition (EU, 2017). In parallel, all GM products that would fall under the parallel legislation on the introduction of GMOs into the environment should be subject to general post-market surveillance, which is largely focused on environmental issues and also includes the observations of potential unexpected or unintended effects in livestock animals by farmers and other professionals in the field (EU, 2001).

The EU-funded MARLON project, which ran from 2012 until 2015 under the Seventh Framework Program for research and technology development, aimed at endowing policy makers with a toolbox that they could provide to applicants who would need to set up post-market monitoring schemes for GM feeds. At the start and throughout the project, such requirement had not been imposed on applicants, though, given that no issues had arisen over the safety and nutritional value of GM feeds during the pre-market safety assessment that could have warranted such monitoring. There was therefore no practical example, whilst it could not be foreseen either for

which particular type of GM feed or under which specific circumstances the requirement could be imposed in future and therefore the methodology to be developed within MARLON had to be generically applicable. Part of the activities of MARLON focused on gathering background information on the possibilities to verify if and how livestock animals have previously consumed GM feeds. This question straddled various aspects to be answered, such as whether the presence of GM ingredients can be verified in feeds, either through the detection of GMO-related DNA or proteins, or through documentation collected in the context of traceability measures. Another question that was to be verified was whether GM-crop-related components such as DNA or protein, could be transferred from feeds consumed and digested by the animal to animal tissues and fluids. The presence of GMO-related DNA or proteins in the latter would then indirectly serve as biomarker of previous exposure of the individual livestock animal (as opposed to feed which would provide an indication of group exposure). Whereas feeding studies with GM feeds that measured health parameters beyond performance do not indicate specific GMO-linked adverse health impacts, this review also considers the possibility as to whether hypothetical changes in the metabolic profile of the livestock animals could be used as an indirect, sub-clinical indicator of exposure for the purpose of linking potential clinical health impacts to prior exposure.

2. GMO DETECTION METHODS

Polymerase chain reaction (PCR) is currently the most commonly used tool for GMO detection and is widely employed within the agri-food industries to test for the presence of GM ingredients. Specifically, quantitative PCR (qPCR) is the method of choice for GMO control routine analysis. Nevertheless, other DNA-based alternative strategies have been developed to more efficiently address the requirements and needs of a growing industry that is producing an increasing number of GM products.

The following sections review DNA-based approaches, databases of methods and complementary screening tools which can serve to confirm the presence of GM crops, not only for the currently approved ones but also for any future crops to be allowed onto the market along the feed and livestock production chains, which may currently still be unauthorized. Finally, several strategies based on detection of newly expressed proteins (NEPs), and only applicable if the genetic modification has an impact at the protein level, are also reviewed.

2.1 IMPACT OF THE FEED MATRIX FOR GMO ANALYSES

Successful DNA amplification is crucial for the detection of specific DNA targets in feeds and in turn, this depends essentially on the capacity of the extraction and purification procedure to obtain high-quality DNA (Cankar et al., 2006; Guo and Zhang, 2013; Holst-Jensen, 2009).

Methods for GMO detection and quantification are usually validated on the basis of certified reference materials or grain materials provided by the respective applicants, while the method in practice will be applied on a wide variety of matrices, generally heterogeneous. Moreover, the feed processing (e.g. grinding, heating, fermentation, extraction using solvent or pressure) may affect the quality and quantity of the extracted DNA (Gryson, 2010). The feed industry uses

a range of raw materials of animal and vegetable origin. The cereals most commonly used for animal diets are maize, barley, wheat and oats, whilst the main sources of protein are meals produced from soybeans, cottonseed and canola. Maize meal is the major component in complete feeds followed by soybean meal (Tisch, 2006). Different DNA extraction methods which are used for food samples may in most cases be successfully employed in feeds samples, but it is important to select the most appropriate DNA extraction methods per feed or matrix and special attention should be paid to sample preparation steps (Berben et al., 2014).

Three performance criteria should be met for adequate GMO analysis:

i) sufficient DNA must be available to guarantee reliable transgene detection at threshold levels as set in the respective legislations
ii) the DNA should not be degraded and of sufficient length to be amplified by PCR; and
iii) the absence of any substance that can cause inhibition of Taq DNA polymerase during DNA amplification (e.g. humic substance, polysaccharides, phenolic compounds, impurities, extraction reagents and other plant secondary metabolites)

The Standard on Foodstuffs EN ISO/IEC 21571:2005 (Nucleic acid extraction) identifies detergent-based methods and commercially available kits that are often used for DNA extraction. A comprehensive review on the influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay was produced by Demeke and Jenkins (2010). The authors also assess advantages and disadvantages of various commercially available DNA extraction kits as well as modifications to published cetyltrimethylammonium bromide (CTAB) methods (Demeke and Jenkins, 2010).

2.2 DATABASES OF GMO DETECTION METHODS AND SCREENING PLATFORMS

In compliance with EU legislation, feed (as well as food) stuffs that contain, consist of, or are produced from GMOs, must be accompanied by documentation stating the GM constituents in the feed product (EU, 2003a; EU, 2003b). The availability of reliable information on methods for GMO detection, identification and quantification is an enabling factor for GMO traceability and, more in general, for the implementation of GMO legislation by official control bodies. All the methods used to identify the EU-authorized GMO have to be provided by the applicants and these methods are introduced in the Compendium of reference methods for GMO analysis (JRC, 2017). An inventory of the available databases providing information on GMO detection methods, as well as the existing platforms and decision support systems developed to facilitate the interpretation of results at screening level, are compiled in Table 1. These are useful tools for laboratories working in the field of GMO detection and contribute to make the analysis of GMOs in the food and feed chain more efficient and cost-effective.

2.3 MULTIPLEX GM EVENT DETECTION

2.3.1 MULTIPLEX REACTIONS APPROACHES

The multiplexing of GMO detection has in recent years been the focus of different research strategies, usually combining a multiplex amplification step with microarray- or fragment length-based identification of the amplified targets. In this case multiplex detection is defined as a number of reactions occurring at the same time in the same tube. Amplification has been based for instance on multiplex PCR, ligation-detection or nucleic acid sequence-based amplification (Chaouachi et al., 2008; Leimanis et al., 2008; Morisset et al., 2008; Prins et al., 2008; Ujhelyi et al., 2012). However, all of these approaches have their setbacks. Bottlenecks were that the required detection limit could not be reached for all targets, or the level of multiplexing was still limited, or the system was not flexible regarding the targets to be detected.

A comprehensive overview of these methods has been previously provided (Fraiture et al., 2015; Pla et al., 2012). A distinction can be made between PCR-based methods and non PCR-based methods. For PCR-based methods, often a distinction is made between oligoplex and multiplex methods, with oligoplex commonly defined as ten targets or less. In this overview several oligoplex assays have been described that do not require more than the standard real-time PCR machine that is already a pre-requisite for a GMO detection laboratory, mostly duplex systems. With additional machinery, such as a capillary electrophoresis system, methods have been described that allow detection of up to nine targets [table 19.1 in Pla et al. (2012)]. Inter-laboratory studies were performed for several of these assays, but full validation has not been reported. A special system, developed by Eppendorf, combining three oligoplex PCR reactions with microarray - based detection was validated through a collaborative trial carried out on reference material in 12 laboratories, in its first version, detecting 15 targets. In all of the systems described, the specific challenges in multiplex PCR, compared to simplex PCR, are:

- More complicated design and optimisation, because conditions cannot be optimised separately for each individual PCR target.
- Competition between the PCR target assays for resources in the reaction.
- Artefacts due to increased complexity, such as primer - dimer formation can result in either reduced sensitivity due to competition effects or even lead to false positive results.

These issues lead to a long development time compared to the reduction in analysis time. For non-PCR based methods such as NAIMA and ligation detection, the highest level of multiplexing was 10, also reviewed in (Pla et al., 2012).

Despite a dedicated work package within an EU-project (Co-Extra) and considerable efforts of many EU research institutes, the techniques described have so far not resulted in a routinely used

multiplex GMO detection system. The most probable reason is that the techniques available at the time simply could not meet the high demands of many targets to be detected combined with potentially highly skewed presence of the combination of targets, despite many attempts at adaptation and optimization. Nevertheless, several oligoplex systems have been described that might be used by individual GMO detection laboratories. The emergence of novel techniques has also contributed to a shift of attention from the techniques described above to the techniques described in the next sections, in the light of multiplex GMO detection.

2.3.2 MINIATURISED MULTI-PCR APPROACHES, INCLUDING DIGITAL (DROPLET) PCR

Multi-PCR approaches are defined within the current text as the parallel execution of PCRs for several different targets within one experiment. Although not strictly multiplex reactions, this does lead to multiplex detection in a given sample in one experimental procedure. In that sense, performing an element screening in a 96-wells plate is already multi-PCR (Mano et al., 2009; Querci et al., 2009; Van den Bulcke et al., 2010). Switching from 96 wells to 384 wells is a first step in increasing the number of targets in the multi-PCR approach.

Still, even further miniaturisation has been achieved in several platforms, moving from microliter to nanoliter scale. The OpenArray system by the Biotrove Company was the first platform for this (Morrison et al., 2006). Another system is the microfluidic dynamic array, in several formats, from Fluidigm (Spurgeon et al., 2008). Both platforms combine thousands of separate reaction chambers in a single device. Both platforms provide quite literally miniaturised qPCR, with real-time detection in every reaction chamber, allowing the user to evaluate the individual amplification curves in much the same way as would be the case for conventional qPCR platforms.

As these systems move from micro- to nanoliter scale, the reaction volume is reduced approximately 1,000 fold. This means that the number of specific DNA targets per reaction, *i.e.* the number of DNA molecules in the sample that contain the fragment to be amplified through the PCR reaction, is also lowered from around ten in conventional qPCR for a single sample near the limit of detection (LOD) to one in 100 reactions (in separate droplets) in these platforms. This means that the number of reactions per target has to be increased from a few to a few hundred to reach the same LOD; of course this greatly diminishes the applicability of these platforms for GMO detection without further modification. An increase in DNA concentration with a factor 1000 is clearly not an option. However, pre-amplification of all the targets is feasible and practiced regularly in reverse transcriptase-qPCR. However, such pre-amplification will likely result in a bias for different targets. This would render GMO quantification impractical. Nevertheless, this pre-amplification can still be useful in a qualitative manner, provided the LOD is reached for all targets (Brod et al., 2014), as a screening tool.

An even further miniaturisation is reached in droplet digital PCR (ddPCR). ddPCR is basically the partitioning of a regular PCR mixture into millions of fractions, typically via water in oil emulsions. In ddPCR, a sample is diluted and partitioned into up to millions of separate reactions so that most contain one or no copies of the sequence of interest. By counting the number of 'positive' partitions, in which the sequence is detected, versus 'negative' partitions, in which it is not, it is possible to determine exactly how many copies of a DNA molecule were in the original sample (Baker, 2012). In a recent study, it has been shown that the methods meet quality criteria as set for GMO methods for routine quantification of GM maize varieties, and in a cost-effective way (Dobnik et al., 2015)

2.3.3 NEXT GENERATION SEQUENCING APPROACHES

One way to overcome the multi-identification issue of (dd)PCR would be to combine it with next generation sequencing (NGS) approaches. The PCR step can be used as a way of efficient library preparation while the sequencing part will result in a more accurate identification of the amplified targets than PCR can ever do. Such sequencing of a selection of known fragments is expected to improve cost-effectiveness of screening methods, already now or in the near future, analogous to disease diagnostic platforms (Valencia et al., 2012).

2.4 DETECTION AND IDENTIFICATION OF UNAUTHORIZED GM EVENTS, INCLUDING UNKNOWN GM EVENTS

2.4.1 THE DEDUCTIVE, OR 'MATRIX' APPROACH BASED ON GM ELEMENT AND GM EVENT SCREENING

The deductive approach to unauthorized GMO (UGMO) detection based on screening of GMO-related elements and specific GM crop events has been described in several research papers (Chaouachi et al., 2008; Dinon et al., 2011; Hamels et al., 2009; Holst-Jensen et al., 2012; Liang et al., 2014; Prins et al., 2008; Querci et al., 2009; Scholtens et al., 2013; Waiblinger et al., 2010). The approach was first outlined in 2001 in the workplan of the European GMOchips research project, (GMOChips, 2002). The basic reasoning is that the presence of any GMO-related DNA element, such as promoters, coding sequences etc., in a mixed sample should be linked to the presence of at least one of the authorized GM specific, or 'event'-specific, sequences, in case only authorized GMOs are present. Conversely, if the presence of a GMO-related element is explained by the presence of an authorized GMO event containing this element, the inference is that an UGMO may be present. In common practice, an element screening is performed first, after which usually a rapid narrowing down of the potentially present GM events is possible. The second step is then to perform the qPCRs for these events, and if the

combination of detected events cannot explain the presence of the combination of detected elements, the presence of a potential UGMO can be deduced. Confirmation of this presence of a UGMO by sequence analysis will be the final step. An example is the site finding PCR approach used for identification of the transgenic Bt rice line KMD1 (Babekova et al., 2009). Several other gene walking strategies have also been described in combination with NGS (Volpicella et al., 2012), though not yet adapted to the specific demands of GMO detection, i.e. a 0.1% detection limit and a multitude of (GMO-related) starting points. Comprehensive overviews of the developments in this area with an emphasis on the detection and identification of UGMOs by gene walking approaches starting from unexplained GMO elements, have been published in recent years (Arulandhu et al., 2016; Fraiture et al., 2014), and the applicability of the approach has been shown in some publications (Fraiture et al., 2015; Liang et al., 2014). These reviews show that there are many strategies that may be informative, but in practice it is still a challenge to select the single strategy that will allow the sequencing of long stretches of unknown DNA adjacent to unexplained GMO-related elements in order to identify unknown GMOs. Also, so far, research in this area has focused on relatively high percentages of GMOs in mixture, or indeed in pure GM materials, whereas the methods of choice in the end will have to be able to also identify low percentages of UGMOs to allow compliance with European legislation.

2.4.2. FUTURE DEVELOPMENTS, FOCUSSING ON NGS APPROACHES

The use of NGS in GMO detection may further allow finding a priori unknown sequences as present in most UGMOs. The most cost-effective way of employing NGS is by the identification of enriched targets rather than by whole genome sequencing (Holst Jensen et al., 2016; Kovalic et al., 2012; Wahler et al., 2013; Yang et al., 2013): the latter is gaining relevance, especially in pure materials, but will not soon be the solution to cover all GMO-related sequences down to 0.1% for all ingredients in a complex mixture. Preferably, in the case of complex samples, enrichment will

be performed by multiplex selection for GMO-related sequences, followed by identification with NGS (Arulandhu et al., 2016). For most of the UGMOs, only limited sequence information, if any, will be known. At the same time, most UGMOs will contain GM elements that can serve as a starting point to 'read' into unknown regions of adjacent, co-introduced and genomic DNA. Whole genome or transcriptome (re)-sequencing would currently be the only option for identification of GMOs that do not contain any previously known GMO element (Holst-Jensen et al., 2016; Tengs et al., 2009; Wahler et al., 2013), in the case of pure raw materials. Whole genome sequencing may work, as indicated, for pure materials when the wild-type genome is sufficiently known and annotated. By in silico subtraction of sequence data that match the non-GM reference genome, or transcriptome, such an approach would yield a subset of the sequence data that can be subjected to de novo assembly and successive further analysis to detect and characterize novel inserts and insertion loci. The many potential genetic differences between sample and reference sequences will, however, not only be related to genetic modifications but also to natural genetic variation in either genome or transcriptome. Also, the amount of sequencing data required is much higher than for the targeted approach. This currently limits this approach for use in samples of very limited complexity in which a high percentage of the UGMO is present.

2.5 DETECTION AND QUANTIFICATON OF NEWLY EXPRESSED PROTEINS (NEPS)

As previously addressed, most NEPs in the GM crops authorized so far in the EU are proteins encoded by the newly introduced genes that confer either tolerance to particular systemic herbicides or resistance to insects. Although expression level of NEPs is variable in the different GM crops and even plant tissues, in general transgenic proteins are present at low concentrations in complex matrices containing, among other constituents, many constitutive proteins that may interfere with protein detection assays.

Therefore, the principal analytical methods used for protein detection and quantification are immuno assays in which antibodies are specifically raised against the NEP and used to specifically bind, separate, and isolate the protein in order to measure its concentration (Lipton et al., 2000).

Among the immuno-based approaches, Enzyme-Linked Immunosorbent Assay (ELISA) has been extensively used for GMO analysis and several ELISA assays have been developed to detect the proteins encoded by various transgenes (e.g. *CP4-EPSPS, Cry1Ab, Cry1Ac, Cry2A, Cry2Ab, Cry3A, Cry9C, pat*) (reviewed in Fraiture et al., 2015). The technique offers several advantages including capacity of automation, good technical performances, easy and rapid screening of numerous samples, low equipment cost, and reduced requirement for amount of reagents. Rapid field variants such as lateral flow strips have also been developed. Moreover, alternative strategies such as portable immunoassay system (Jang et al., 2011) and immune PCR method were also used to identify GMO (Allen et al., 2006; Santiago-Felipe et al., 2014). Among other protein-based methods, mass spectrometry-based technology was also proposed to characterize GM plants (García-Cañas et al., 2011).

Despite the previously mentioned advantages such as rapidity and simplicity, applicability of these methods is limited to raw or partially processed materials. Proteins are highly degraded or denatured by food processing and changes in the structure of the targeted proteins make reliable immunological detection difficult (De Luis et al., 2009; Margarit et al., 2006). Therefore, monitoring for GM products in processed food and feed relies more routinely on DNA-based detection methods (Alderborn et al., 2010).

3. FEASIBILITY OF MEASURING GMO-RELATED DNA AND PROTEINS IN ANIMAL SAMPLES

The detection and fate of GMO-related DNA and/or newly expressed proteins in animal food has been highlighted as an important issue in the continuing debate over the use of GM crops in livestock feed as well as human food products. When tracking the fate of such GM feed (e.g. made from seeds or forage) and its GMO-related components, the specific focus is set on traceable GMO-related DNA and NEPs present in GM feed. Current detection technologies enable a very effective analysis of DNA traces, whereas the presence of newly expressed proteins is, due to degradation and/or biotransformation, not so easily detectable in animal samples.

As stated in the introduction, detection of GM plant components (specifically transgenes and/or newly expressed proteins or metabolites) in animal samples could in theory also provide a means of assessing an animal's previous exposure to GM feed. Dietary DNA is not completely degraded in the gastrointestinal (GI) tract and DNA fragments can be absorbed and transferred to various organs and tissues. Einspanier et al. (2001), for example, detected fragments of non-GM DNA from feed in some tissues of various domestic animal species. Since then, numerous studies investigating the fate of ingested GMO-related DNA and NEPs in animals fed commercialized GM plants have been reported (reviewed by Blair and Regenstein, 2015; Einspanier, 2013; Snell et al., 2012; Swiatkiewicz et al., 2014).

This section evaluates the feasibility of measuring GMO-derived DNA or proteins in animal samples to determine whether an animal may have consumed GM feed.

3.1. TRANSFER OF GM DNA FROM FEED INTO ANIMAL TISSUES

3.1.1 DIGESTION AND FATE OF DIETARY DNA

Dietary intake of GMO-related DNA depends on a number of factors ranging from the type of feed, the processing of feed, the amount of feed ingested, as well as the amount of such DNA present in the feed. Ingested DNA is broken down during both the mechanical and enzymatic processes of digestion and GM crops are digested by animals in the same way as conventional crops. A number of investigations in different animal species have determined that dietary DNA is almost completely hydrolyzed after passage through the duodenum (Jonas et al., 2001), while the passage of plant DNA fragments, particularly small fragments of the high-copy chloroplast *rubisco* gene, across the intestinal barrier has been stated as a natural event, whilst they are unlikely to be incorporated into animal products at substantial levels (reviewed by Alexander et al., 2007). Whilst low-copy genes such as GMO-related genes are unlikely to persist in ruminants until reaching the proximal small intestine due to ruminal and intestinal DNase activity, DNA fragments derived from dietary DNA have been detected throughout the entire digestive tract in non-ruminants, but they consist of small fragments (less than 400 bp, rarely up to 1700 bp) and detectability correlates with a high intake of the target DNA (reviewed by Alexander et al., 2007; Einspanier and Flachowsky, 2009, Rizzi et al., 2012). This DNA fragmentation and variable detectability of low-copy genes, plus the limited time of intestinal passage, raises doubts about the applicability of detection of DNA in digesta of a livestock animal as a measure of the history of its prior exposure to a particular GM feed ingredient.

3.1.2 DETECTABILITY OF GMO-RELATED DNA IN ANIMAL SAMPLES

A literature search to explore the feasibility of detecting GMO-related DNA in animal samples from animals that have consumed GMOs has been carried out (Tables 2-3, Supplementary Material S1). Early studies in mice by Schubbert et al. (1998, 1997, 1994) investigating the fate of DNA in the GI tract of mammals showed that foreign DNA is not completely degraded in the GI tract and that DNA fragments can be taken up through the intestinal wall then carried in various

organs and tissues. Numerous other feeding studies since have also examined the fate of dietary DNA in a range of different animal species fed with GM crops.

<< place Table 1 and Table 2 about here >>

General Overview

Eighty-three studies focusing on detection of foreign DNA in animals were reviewed (Tables 2-3). Seventy-three out of these 83 studies focused on DNA detection in various livestock species [i.e., cattle (21), poultry (19), pig (18), fish (6), sheep (4), rabbit (3), goat (1), and deer (1)]; and ten on DNA detection in non-livestock species (i.e. mouse (6), rat (2), zebrafish (1), wild boar (1) and human (1)]. Thirty five of the studies carried out in livestock detected GMO-related DNA and 37 studies were unable to detect such DNA). Out of the ten studies in non-livestock species, five did not test for the presence of GMO-related sequences, three detected such sequences and two did not. All of the studies used PCR as the primary test method for detection and included controlled feeding trials.

Tables 2 and 3 offer a summary of these studies and include information regarding: 1) the type of study, 2) GM plant or material used 3) animal species studied 4) tissues sampled, 5) detectability of GM and/or plant reference DNA in different tissues, and 6) size of the fragments analyzed for and those that were indeed detected. Taking into consideration all the studies, the most frequent tissues, fluids and products assessed were GI tract content, blood, and muscles; and also milk in the case of dairy cows. Figure 1 summarizes the overall results reported, considering the most relevant samples per animal species. It is noteworthy that more than 80% of samples were tested for the presence of both the transgene and plant reference gene (control) and GMO-related DNA was detected in GI-tract samples of all livestock species evaluated. Lower percentage of positive detection of any transgene was observed in blood, and muscle samples. Overall, findings indicate that dietary DNA is not completely broken down during feed processing and digestion and that fragments are able to be detected by PCR in various organs

and tissues. Although there are a number of studies looking into the detection of dietary DNA in animals, the variation in their study design and methods used makes it difficult to compare results. A comprehensive review describing the results reported for each different livestock animal species was carried out and presented below.

<< place Figure 1 about here >>

Ruminants

Poms et al. (2003) investigated the fate of plant DNA by an intravenous application of purified plant DNA and feeding study. After the intravenous application, a fast elimination of marker DNA in blood or its reduction below the detection limit was observed. Furthermore, after the feeding trial, blood, milk, urine, and feces of dairy cows were examined, and foreign DNA was detected by amplifying a fragment of the maize *invertase* gene and a fragment of the soya *lectin* gene. No specific DNA transfer from feeds into milk was detectable.

Phipps et al. (2003) studied the fate of plant DNA in lactating cows fitted with ruminal and duodenal cannulas. Multicopy genes (*bovine mitochondrial cytochrome b* and *rubisco*) were detected in the majority of samples analyzed in both the liquid and solid phases of ruminal and duodenal digesta, milk, and feces, but rarely in blood. Single-copy genes (soy *lectin* and *maize high-mobility protein*) were only detected in the solid phase of rumen and duodenal digesta.

A study carried out on cattle and lactating cows fed with the GM insect-resistant maize or conventional maize indicated that only short DNA fragments derived from plant chloroplasts could be detected in blood lymphocytes (Einspanier et al., 2001). Plant DNA was not found in all other cattle organs investigated (muscle, liver, spleen, kidney), except for trace amounts in milk.

The influence of GM insect-resistant CBH351 (Starlink) maize on the transfer of the *cry9 c* gene to different tissues (blood, liver and muscles) was examined, and compared with a diet containing

non-transgenic control maize (Yonemochi et al., 2003). The *cry9 c* gene was not detected in any tissue at the end of the experiment.

To address the transfer of *cry1Ab* gene to animal tissues, Chowdhury et al. (2004) tested the presence of recombinant *cry1Ab* gene on calves fed with GM insect-resistant Bt11 maize in a sub chronic 90-day feeding study. Recombinant *cry1Ab* genes were not detected in peripheral blood mononuclear cells or visceral organs.

Persistence of plant DNA sequences in the blood of dairy cows fed with GM insect-resistant (Bt176) and non-GM conventional maize silage was investigated by Bertheau et al. (2009). No samples tested for transgenic sequences (35S promoter and Bt176-specific junction sequence) were positive. Only faint punctual positive results occurred randomly and were probably due to post sample collection or laboratory contamination or can be considered as artefact as they were not confirmed.

Several feeding trials were carried out to analyze the transfer of plant DNA and GMO-related DNA to milk. Studies carried out by Einspanier et al. (2001) and Phipps et al. (2003) detected fragments of naturally occurring multicopy plant genes in milk samples, but not GMO-related DNA. Data from 60 multiparous Holstein cows from a 12-week continuous design feeding trial (Phipps et al., 2005) showed that none of the 90 milk samples tested positive for either GMO-related DNA (event T25) or the single-copy endogenous *Zea mays* gene, *alcohol dehydrogenase*. GMO-related DNA was not detected in milk of eight cows fed with silages from GM maize containing two newly introduced genes, *i.e.* the herbicide tolerance-conferring *mepsps* and insect resistance-linked *cry1Ab* genes (Calsamiglia et al., 2007). Guertler et al. (2009) analyzed 90 milk samples collected from cows fed either with rations containing GM insect-resistant maize carrying the *cry1Ab* gene (n=8) or non-GM maize (n=7) for six months. The *cry1Ab* gene was not detected in any of the analyzed samples.

Only one study reported the detection of GMO-related DNA in milk (Agodi et al., 2006). The study, which was carried out on milk samples collected from the Italian market, was able to detect maize and soybean GMO-related DNA. However, such traces of GMO-related DNA was interpreted as an indicator of fecal or airborne contamination with feed DNA or feed particles. Finally, in a study carried out in real life husbandry systems to evaluate the carry-over of GMO-related DNA in milk, neither transfer of transgenic nor single copy plant DNA was determined (De Giacomo et al., 2016).

Sharma et al. (2006) investigated the persistence of plant derived and GMO-related DNA in sheep fed with GM Roundup Ready canola meal. The study showed that the feed-ingested DNA fragments (endogenous and GMO-related) do survive towards the terminal gastrointestinal tract and that uptake into gut epithelial tissues does occur although transmittance to visceral tissue was not confirmed.

The fate of GMO-related DNA in sheep was investigated by Alexander et al. (2006). The study evaluated the presence of GMO-related DNA in digesta, feces and blood samples collected from six ruminally and duodenally cannulated sheep fed with forage-based diets containing 15% Roundup Ready rapeseed meal. GMO-related DNA was only detected in ruminal and duodenal fluids.

In a study carried out on 53 ewes and their progeny fed with a diet including GM insect-resistant Bt176 maize for three years, GMO-related DNA was not detected either in blood, ruminal fluid or ruminal bacteria (Trabalza-Marinucci et al., 2008).

Rabbit

Tudisco et al. (2010) monitored the detection of two DNA plant sequences [i.e. high copy number chloroplast genes from barley (*trnI;*100-bp amplicon, multi-copy gene) and *lectin* reference gene from soybean (118-bp amplicon; single-copy gene)] in different tissues (i.e. blood, liver, kidney, spleen, muscle and digesta) of rabbits fed with a diet including barley grain (15%) and soybean meal (12%). The chloroplast fragment detection frequency was higher in muscle (90%), liver (80%), kidney (80%) and spleen (80%) than in blood (40%) and digesta samples. Moreover, chloroplast DNA was found in 40 and 30% of duodenum and caecum contents, respectively, and in 30% of feces. The soybean *lectin* gene was not detected in animal samples.

Morera et al. (2016) assessed the possible DNA transfer from GM feed to rabbit tissues in samples of blood, liver, kidney, heart, stomach, intestine (jejunum), muscles, and adipose tissue collected from rabbits fed with GM soybean meal. All samples were analyzed, by real-time PCR, for the GM Roundup Ready soybean and for the endogenous *lectin* gene. No fragments of GMO-related DNA and *lectin* gene were detected in tissue of rabbits except in the DNA extracted from stomach digesta, feces and hair. However, the presence of traces of transgenic soybean in hair samples was explained by an environmental contamination.

Poultry

Einspanier et al. (2001) investigated the presence of maize and GMO-related DNA in eggs and tissues (i.e. muscle, liver, spleen, kidney) in broiler and laying hens fed with GM insect-resistant- and non-GM conventional maize, A fragment of a chloroplast gene was detected in different tissues, but no foreign plant DNA fragments were found in eggs. Bt-gene specific constructs originating from GM insect-resistant maize were not detected either in tissues or eggs.

Rossi et al. (2005) investigated the fate of feed-derived DNA (plant-specific genes *zein* and *Sh-2*, and *cry1Ab* gene) in the digestive tract of broilers fed with GM maize. Detection frequency of *zein* gene (high copy number gene) was high but significantly decreased in distal sectors of the digestive tract. A fragment of the *cry1Aab* gene, corresponding to the minimal functional unit, was detected only in gizzard of birds fed with GM maize. *Sh-2* was detected only in gizzard of all broilers. Blood samples were positive with low frequency only for *zein* analysis. No significant difference in DNA detection was observed between birds fed with GM- or isogenic maize, confirming that feed-derived GMO-related DNA undergoes the same fate as isogenic feed-derived DNA. Aeschbacher et al. (2005) carried out a study on broiler and laying hens fed with two different diets containing either 60% conventional or 60% Bt176 maize. The presence of target DNA was investigated in tissues of muscle, liver, spleen and eggs. The maize-chloroplast *ivr* gene fragment was amplified in all samples; no GMO-related plant DNA fragments were amplified in samples from tissues from animals fed the Bt176-maize containing feed. Rehout et al. (2008a) performed three feeding experiments on broilers fed with GM Roundup Ready soybean. In total, 118 blood samples were analyzed and the presence of DNA fragment was reported in 18 samples: seven samples were positive for control gene *lectin* and transgene sequences were identified in 11 samples.

A similar study (Rehout et al., 2008b) was performed on broilers fed with: maize MON810 (first group), GM Roundup Ready soybean (second group), Roundup Ready soybean and maize MON810 (third group), and a GMO-free diet (fourth group). Fragments of soybean transgene were identified in three samples of liver. On the contrary, fragments of GM-maize-related DNA were not detected in livers. There was no positive detection of any transgene in kidney samples. Comparable results were obtained from two multigenerational studies on quail (Flachowsky et al., 2005; Korwin-Kossakowska et al., 2013).

The fate of GMO-related DNA from GM insect-resistant maize and GM herbicide-tolerant Roundup Ready soybean meal in broilers fed feeds containing ingredients derived from these GMOs was also investigated by Swiatkiewicz et al. (2010). In a 42-day floor pen experiment, broilers were fed with maize and soybean meal diets (55%-60% and 32%-37% respectively). DNA was extracted from the gizzard, duodenum, jejunum, ileum, and caecum digesta, excreta, and in blood, liver, spleen, and breast muscle. The obtained data indicated that GMO-related DNA sequences from GM insect-resistant maize and Roundup Ready soybean are well digested in the gastrointestinal tract and are not transferred to broiler tissues.

A feeding trial was conducted by Ma et al. (2013) to assess the fate of GMO-related DNA in digesta, blood, tissues, and eggs. Fifty-week old laying hens (n = 144) were fed with a diet containing 62.4% of phytase-transgenic GM maize or with non-GM isogenic maize for 16 weeks. The GMO-related *phyA2* gene was rapidly degraded in the digestive tract and was not detected in blood, heart, liver, spleen, kidney, breast muscle, and eggs of laying hens, indicating that there was no evidence of *phyA2* gene translocation to tissues of laying hens.

In conclusion the studies on the fate of transgenic and plant DNA in poultry showed that DNA is not completely digested in the gastrointestinal tract and that chloroplast DNA can be found in poultry organs. However, no foreign plant DNA fragments were detected in eggs.

Pigs

Chowdhury et al. (2003a) examined the presence of *cry1Ab* gene, in the gastrointestinal contents of five pigs fed with GM maize Bt11 and five fed with non GM maize. Fragments of recombinant *cry1Ab* gene were detected in the gastrointestinal contents of the Bt11-fed pigs but not in the control pigs. C*ry1Ab* gene fragments were not detected in the peripheral blood.

Reuter and Aulrich (2003) investigated the passage and fate of ingested DNA in 48 pigs fed with diets containing parental or GM insect-resistant maize. GMO-related or maize-specific DNA was not detectable in tissue samples of pigs.

In the paper by Mazza et al. (2005), the potential transfer of diet-derived DNA to animal tissues after consumption of GM plants was assessed. Blood, spleen, liver, kidney and muscle tissues from piglets fed for 35 days with diets containing either GM (MON810) or a conventional maize were investigated for the presence of plant DNA. *Zein* was present in all the examined tissues except muscle, and a fragment of the *cry1Ab* transgene was detected in blood, liver, spleen and kidney. The intact *cry1Ab* transgene or its minimal functional units were never detected.
Sharma et al. (2006) investigated the persistence of plant-derived GMO-related DNA in pigs fed with diets containing 6.5 or 15% GM Roundup Ready canola. Native plant DNA and the *cp4epsps* transgene were tracked in cecal content and tissue from the duodenum, caecum, liver, spleen, and kidney of pigs. High-copy chloroplast-specific DNA was detected in all digesta samples, the majority (89-100%) of intestinal tissues, and at least one of each visceral organ sample. Each of the five *cp4epsps* transgene fragments (179-527 bp) surveyed was present in at least 33% of porcine cecal content samples (maximum=75%). The feed-ingested recombinant DNA was not detected in visceral tissues or in the spleen from pigs. Only, one liver and one kidney sample from the pigs (different animals) were positive.

The digestive fate of *Escherichia coli* glutamate dehydrogenase (*gdhA*) DNA from transgenic maize in diets fed to weanling pigs was investigated by Beagle et al. (2006). Weanling pigs were fed with a diet containing 58% *gdhA*-maize for one week. GMO-related DNA (490-bp amplicon) was detected in 71.43% of the stomach and 1.79% of the ileal ingesta samples from treatment animals but, was not detected in the large intestine, white blood cells, plasma, liver, or muscle

samples. These data suggest that the targeted GMO-related DNA started undergoing degradation in the stomach and was undetectable in the large intestine.

The study of Walsh et al. (2011) on the effect on immune responses and growth in weanling pigs determined also the fate of GMO-related DNA *in vivo*. The detection of the *cry1Ab* gene was limited to the gastrointestinal digesta and was not found in the kidneys, liver, spleen, muscle, heart or blood. Analogous results were obtained by Swiatkiewicz et al. (2011), Walsh et al. (2012) and Sieradzki et al. (2013) describing that GMO-related DNA was detectable in the content of the stomach and duodenum but not in blood and other examined organs.

In conclusion, most of the studies performed on pigs suggest that feed-ingested DNA fragments (endogenous and transgenic) are detectable in the gastrointestinal tract. A very low frequency of transmittance to visceral tissue was reported (Sharma et al., 2006), and one article out of 18 (Mazza et al., 2005) detected a small GMO-related DNA fragment in blood, liver, spleen and kidney.

Fish

Studies with fish have produced similar results to those obtained with other livestock animals. Sanden et al. (2004) focused on the fate of selected GM soybean DNA fragments from feed to Atlantic salmon, concluding that transgenic sequences (120 and 195 bp) and the *lectin* gene (180 bp) could be detected in GI tract samples. Moreover, the study also measured the transference of GMO-related DNA to a variety of fish tissues, but neither transgenic nor conventional soybean DNA fragments could be detected in liver, muscle or brain tissues resected from sacrificed fish. The same research group measured the concentration of GMO-related DNA (low copy DNA) and plant DNA (multicopy DNA, *sRubisco*) in intestine samples collected from Atlantic salmon fed with diets containing GM or non GM soybean (Sanden et al.,

2011). GMO-related DNA was not detected, while the DNA of the plant reference gene *sRubisco* was detected in several of the intestine samples.

In rainbow trout fed with GM soybean meal, a fragment of *CaMV35S* promoter (220 bp) was detected in the contents of digestive system, head, kidney and muscle only of fish fed the GM diet; it was not detected on the fifth day after changing the diet to one containing non-GM soybean meal (Chainark et al., 2006, 2008). Interestingly, chloroplast DNA (257-bp amplicon_ was detected in white blood cells and spleen of some (≤15%) of the fish fed either diet (Chainark, 2008).

3.1.4 CONSIDERATIONS AND LIMITATIONS

DNA extraction method: Numerous studies involving plant material indicate that DNA amplification and detection efficiency is dependent upon the DNA extraction method used (Cankar et al., 2006; Holst-Jensen, 2009; Zhang and Guo, 2011). Since the majority of DNA extracted from animal tissues is likely to be of animal origin and only a small percentage is likely to be of dietary origin, there is also a need for efficient DNA extraction methods from animal tissues. A study by Nemeth et al. (2004) examining three extraction methods (silica gel purification, CTAB precipitation, and chloroform extraction) used for cow, chicken, and pig muscle as well as bovine milk samples, showed chloroform extraction to have the best results. Outside of this study, there is a lack of data regarding DNA extraction methods in animal tissue. The International Organization for Standardization (ISO) has published guidelines (ISO 21571; *cf*. ISO, 2005) for DNA extraction from plant tissue which highlights, along with numerous studies, the importance of validation of extraction methods (Zhang and Guo, 2011).

- **Gene copy number** may also affect detection of plant DNA sequences in animal tissues. Foreign plant DNA fragments should be detectable in animal samples if the amount of starting

material is high enough. This is particularly relevant with respect to chloroplast genes as these are often used as the control or comparison in studies examining the detection of plant-derived, GMO-related DNA in animal tissues. Due to its higher abundance within the plant genome (500 to 5000-fold higher than in chromosomally located genes), the chloroplast gene may be more easily detectable and therefore not suitable as a control or comparison (Beever et al., 2003; Jennings et al., 2003b). Additionally, Klaften et al. (2004), reports that chloroplast DNA is more easily aerosolized than nuclear DNA and therefore, under typical laboratory conditions, has the potential to contaminate DNA extraction or PCR reaction buffers, leading to increased detection.

- **Fragment size** of dietary DNA as well as time after ingestion also influences detection. In general, fragment size decreases along the GI tract, with mostly short fragments (≤ 200 bp) remaining in the lower colon and feces (Nemeth et al., 2004; Rizzi et al., 2012). Size of the DNA fragment may also affect absorption into tissues and organs other than the digestive tract (Nielsen et al., 2005). A study by Klotz et al. (2002) found evidence of a size dependent effect of fragment absorption as none were positive for a chloroplast sequence of 532 bp but all were positive for a smaller sequence consisting of 199 bp. Using short amplicons should increase the chance of detecting highly degraded and diluted DNA in complex samples.

- **Contamination** can also be a source of false positives when testing for GMO-related DNA. A study by Klaften et al. (2004) showed when performing PCR under standard laboratory conditions, 48% of tested bovine samples were positive for a chloroplast gene in comparison to three percent of the same samples, which tested positive when working under stringently controlled laboratory conditions. Therefore, it is important to observe strict protocols to avoid contamination, especially with the use of highly sensitive PCR procedures, and to use positive and negative controls throughout an experiment to test for possible contamination related errors

(Klaften et al., 2004). Other potential causes of contamination can occur during the sampling of organs and during milk sampling. For example, when sampling organs, unintended puncture of the GI tract can lead to contamination of other tissues and internal organs (Nielsen et al., 2005); when obtaining milk samples, it can be difficult to collect samples free of environmental contamination from plant-derived dust and material (Agodi et al., 2006; Nemeth et al., 2004). Another source of contamination or misinterpretation may be the fact that particularly tissues and organs with blood supply could contain white blood cells both within the blood vessels or migrating through extravascular spaces, since white blood cells (from blood) have previously been found to contain fragments of e.g. chloroplast DNA from ingested plant materials (e.g. Klotz and Einspanier, 1998)

- **Standardization and guidelines**: EFSA and the International Life Sciences Institute have established best practice protocols for livestock feeding studies (EFSA, 2011b; ILSI, 2003). However, standardization and establishment of international guidelines is greatly needed for studies assessing detection of GMO-related DNA in animal tissues. Existing studies focusing on detection of plant DNA in animals are difficult to compare as they involve a range study designs and approaches using different animal species at different production stages, different plant lines and feed types, as well as different laboratory and testing protocols.

3.1.5 CONCLUSION

Since the first investigations focusing on the digestive fate of DNA in animals were carried out in the 1980s, many studies have been undertaken to assess the fate of foreign DNA in animal tissues. The majority of studies were designed as short-term feeding studies, but a few articles investigated the fate of GMO-related DNA in animal samples obtained from long-term feeding trials although they do not generate significant new information. As it should be expected, a number of studies have illustrated that dietary DNA is not completely degraded in the GI tract

and that DNA fragments can be absorbed through the intestinal wall and deposit in various organs and tissues. Transfer of DNA has been observed to some extent with e.g. relatively abundant DNA from chloroplasts of plant cells. Transgenic DNA has been detected in faeces and GI tract but occasionally in animal fluid and organ samples. No transgene DNA has been reported in animal-derived edible products such as milk or eggs. Furthermore, it can be concluded that there are no standardized guidelines and studies with regards to the sampling of animal tissues and related detection of plant-derived, GMO-related DNA in these tissues.

3.2. DETECTABILITY OF TRANSGENIC PROTEINS IN ANIMAL SAMPLES

Western blot analysis and lateral flow test strips were used for the *in vivo* investigation of digestive fate of the Cry3 Bb1 protein in laying hens fed with diets containing GM MON863 maize (Scheideler et al., 2008). Small amounts of protein or protein fragment(s) were detected only in digesta and feces but not in hepatic or breast muscle tissues; eggs gave inconclusive results because of the presence of interfering substances. Western blot analysis were also applied in experiments with laying hens fed with GM maize containing transgenic phytase, showing that the phyA2 protein is rapidly degraded in the digestive tract and is not detectable in birds' tissues or eggs (Ma et al., 2013).

Quantitative ELISA methods were used in the following studies on pigs and cows. GMO-related Cry9C proteins were not detectable in blood, liver or muscles of pigs fed with a diet containing GM insect-resistant StarLink maize (event CBH 351) expressing this NEP (Yonemochi et al., 2010); Walsh et al. (2011) determined the presence of transgenic Cry1Ab protein only in the gastrointestinal digesta, not in the tissues (kidneys, liver, spleen, muscle, heart or blood) of weanling pigs fed with MON810 maize. No Cry1Ab-specific antibodies were found in sows or offspring in the experiment by Buzoianu et al. (2012) who investigated the effect of feeding GM

MON810 maize to sows during gestation and lactation on the fate of GMO-related products in tissues of sows and their offspring.

Paul et al. (2008) analyzed 20 plasma samples from cows (n=7) fed with non-transgenic maize and 24 samples from cows (n=8) fed with GM maize (collected before and one and two months after feeding) for the presence of the Cry1Ab protein. No plasma sample was positive for the presence of the Cry1Ab protein. The same investigators reported that transgenic Cry1Ab protein from MON810 maize is increasingly degraded during dairy cow digestion, thus its relative amount in feces is markedly reduced, indicating that Cry1Ab protein is not more stable than other feed proteins (Paul et al., 2010). Results from a 25 month long-term feeding study revealed that immuno-reactive Cry1Ab protein fragments are not transferred to body fluids as urine, blood plasma and milk of cow fed with GM insect-resistant maize expressing this NEP (Guertler et al., 2010, 2009), however, as the protein is not completely degraded by digestive processes in the gastrointestinal tract the Cry1Ab is still detectable in feces. Singhal et al. (2011) analyzed the GMO-related Cry protein levels in lactating multiparous cows fed with GM insect-resistant (Bollgard II®) or non-genetically modified isogenic cottonseed. The results indicated that Cry proteins were not detected in milk or plasma samples. Similar results were obtained by Mohanta et al. (2010).

3.2.1 CONSIDERATIONS AND LIMITATIONS

- **Concentration:** *In vitro* digestibility tests such as those performed on purified NEPs, using simulated gastric and/or intestinal fluids (e.g. pepsin and/or pancreatin) in particular conditions, showed that the great majority of NEP in GM crops authorized so far were extensively and rapidly degraded by proteolytic enzymes and therefore are expected to be degraded during ruminal fermentation as well as gastric and intestinal digestion. Peptidic fragments that may be absorbed during the digestive process should be identified and assessed to confirm they are

likely metabolized and that the probability to detect any eventual residual trace (still functionally active or antigenic) of protein or fragments in animal tissues, products, or urine is very low.

- **Specificity:** The specificity of the antibodies used for detection and quantification of NEPs in GMOs is crucial for the validity of the assay. Antibodies are usually raised against the native and functionally active form of the NEP as it is expressed in the plant. It is important to note that small modifications in the structure of the protein (e.g. due to technological processing, digestion/absorption/storage and elimination by the animal) would impair the recognition and binding by antibodies and therefore preclude detection.

- **Matrix interferences:** Interferences with the food/feed matrix or with components of a complex biological environment, such as animal tissues/fluids or excreta, might impair the assay (Scheideler, et al., 2008).

Therefore, it seems unlikely that analytical methods such as immuno-assays can reliably indicate whether an animal has been exposed to an authorized GM plant through detection of protein traces or fragments in tissues, biological fluids and animal-derived products. GMO-related-protein detection can only be achieved for certain GM plants and intake levels, in digesta and feces samples.

3.3 CONSIDERING A METABOLOMIC APPROACH TO INDIRECTLY DETECT GM-CROP EXPOSURE

Metabolomics refers to the systematic identification and quantification of metabolites of a biological system and aims at detecting changes in the dynamics and balance of metabolic pathways through a comprehensive analysis of the pool of endogenous metabolites present in a biological medium when a perturbation of the metabolic network arises. So far it has mostly

been used in plants to evaluate the effect(s) (intended or unintended) of a genetic modification as a complementary study of the compositional analysis (i.e. conventional chemical analysis) of the raw material, hence measuring a much higher number of analytes in a non-hypothesis driven mode. This should be understood as the first step of the more general frame of assessment and the best candidate for filling the gap between animal feeding studies and analysis of the biological impact resulting from insertion of a gene in the genome (Heinemann et al., 2011; Ricroch, 2013 and references therein). Various recent comprehensive studies on a wide range of varieties of crops grown in different environments indicate that environment and genotype are the main factors influencing metabolome composition (*e.g.* Harrigan et al., 2015; Kusano et al., 2015; Chen et al., 2016; Tang et al., accepted for publication), confirming previous observations by Ricroch et al. (2011) in their review of prior omics studies performed on GM crops and their comparators. Moreover, differences observed between GM and non-GM comparators can be associated with conventional back-crossing practises (Harrigan et al., 2016).

The second step should be the search of specific metabolic deviations in animals fed a GMO-based diet in order to determine whether it is of toxicological or physiological relevance that would reflect a necessary adaptation of the metabolism. In principle, metabolomic studies of the animal fed a GMO-based diet may be an approach that could provide information on the exposure of an animal to a GMO-based diet. Changes in animal metabolomes may be correlated to changes in the metabolome of the plant incorporated in its feed, if these changes persist after feed formulation, although no direct causality has been shown to exist between both. If no significant change in the chemical composition of the plant results from the genetic modification (e.g. herbicide-tolerant or insect-resistant GM crops) and, if the nutritional value of the GM crop and its conventional comparators are similar, no qualitative or quantitative impact on the animal production performance is expected. On the contrary, in very particular cases, if

the intended effect of the genetic modification is to significantly change the composition of the plant (e.g., high-oleic-acid- or stearidonic-acid-rich soybean), consumption of feed with high levels of the altered GM-crop derived feed component, may in exceptional cases result in measurable changes in the animal products (meat, milk, egg), or even in the growth/breeding/production performances of the animal consuming this feed. Therefore, we can anticipate that such a GM-crop-based feeding could then be identified by a seemingly targeted analysis of body/tissue composition. In more general terms, if the feeding regimes do not specifically aim for such changes in animal product composition or performance, such effects will be highly unlikely as individual GM crop components will only constitute minor parts of the feeding materials consumed by individual animals. Other factors are therefore likely to contribute more to changes in the animal's metabolomics profiles. In animals as well as humans, metabolomics may detect early biomarkers of biological effects but also markers of exposure after administration of a toxicant, a drug, or even after a change in the diet or initiation of a pathological insult. This explorative approach is mainly encountered in a well-designed experimental context, and more rarely, in an epidemiological one.

No metabolomic studies have been published concerning alteration of the physiological response of a laboratory animal model fed a GMO-based diet *versus* a conventional one, whilst the recently (2017) concluded GMO90plus project has investigated the urine, organs, and blood of rodents that received diets containing GM herbicide-tolerant maize or conventional counterparts (GMO90plus, 2015). In an earlier, preliminary study urine samples from rats fed GM potatoes were subjected to metabolomics analysis with nuclear magnetic resonance. These GM potatoes, which had been analyzed with mass spectrometry and infrared spectroscopy as metabolomics tools, had been rendered resistant to Potato Virus Y or expressed the *Nia2* nitrate reductase gene, the latter resulting in a more homogenous size distribution of tubers. This study established correlations between the plant and animal urine metabolomes through a linkage

between the variances of these two sets of data, considering also the hierarchy in predominant variation factors including cultivar and year of cultivation (Paris et al., 2004; Paris, 2006).

In general, the influence of confounding factors (e.g. feed variations, age and gender of animals, temperature, season, production performances, reproductive stages, stage of lactation or growth, breed, and husbandry practices) on the characterization of valuable biomarkers of GM-crop-based feeding exposure needs to be extensively evaluated to assess whether metabolomics-related methods may, in exceptional cases, be useful to indirectly detect an exposure in farm animals in the context of epidemiological studies.

4. SUITABILITY OF THE GMO TRACEABILITY DOCUMENTARY SYSTEM TO RECORD GMO CONSUMPTION BY ANIMALS

Whilst the options to measure prior consumption of a given feed by livestock animals obviously involve chemical/biochemical/ and molecular-biological analytical methods, the EU has also imposed traceability requirements for GMOs involving record keeping, which merit further exploration for their applicability to measure consumption of GMOs in the frame of epidemiological studies. Since the introduction of GM crops and derived feed ingredients on the European market the EU has established a legal framework to ensure labelling and traceability of GMOs (European Commission Regulations (EC) No 1829/2003 and No 1830/2003) (EU, 2003a; EU, 2003b). Compliance with these Regulations results in extensive documentation accompanying GM materials throughout the feed chain, focusing for the larger part on the GMO-derived character of feeding materials, rather than on the identification of individual GMOs. Here we envisaged using the available information to determine livestock exposure to GMOs.

Within the EU, the vast majority of cultivated GM crops are produced in Spain: In regions such as Catalonia, GM maize (i.e. the only GM crop authorised for cultivation in the EU) represents about two-thirds of cultivated maize. Virtually all produced GM maize is processed as a feed ingredient. At the same time, Catalonia has a strong livestock farming sector. Here we used Catalonia as an example to study the suitability of using the implemented documentary GMO traceability system to assess GMO intake by animals.

The following sections briefly address the EU regulations regarding labelling and traceability of GMOs; and provide a specific example to question the suitability of using the implemented documentary GMO traceability system to infer GMO intake by animals.

4.1 REGULATION

Since the introduction of the first GM plant varieties on the European market in 1996, the EU has established a legal framework in order to ensure that not only the development of this technology takes place in complete safety but also its release into the environment and its commercialisation. Legislation on GMOs includes, among others, Regulation (EC) No 1829/2003, covering food and feed applications of products consisting of, containing, or produced from GMOs, and Council Directive 2001/18/EC, regulating field cultivation and other forms of environmental release of GMOs (e.g., import and processing). New recommendations were issued in 2010 that allow Member states to regulate cultivation of GM crop on their territories.

4.1.2 LABELLING

European Commission Regulations (EC) No 1829/2003 and No 1830/2003 give instructions for GMO labelling. Labelling was introduced to give consumers the freedom of choice (i.e. allowing the decision between products from different agricultural systems). All products need to be labelled if they contain or consist of GMOs, or if they are produced from or contain ingredients produced from GMOs. During production, transport and processing admixture of small amounts of crops from different origins is considered unavoidable. The labelling regulation sets a threshold for accidental presence of GMOs which attempts to be equilibrium between producers' and consumers' requests. This threshold is 0.9% for each crop that is used to produce the final product, provided that this presence is adventitious or technically unavoidable. The threshold is only applicable with relation to EU-approved GMOs, whilst for GM varieties that are not approved, no threshold has been established.

4.1.2 TRACEABILITY

According to Regulation (EC) No 1830/2003, traceability means the ability to trace GMOs and products derived from GMOs at all stages of their placing on the market through production

and distribution chains. Traceability rules apply to products consisting of, or containing, GMOs, and food and feed produced from GMOs. Traceability requires that anyone who introduces a GM product into the market must provide information on the unique identifier for this GMO to those who are next in the supply chain. Information with relation to the presence of GMOs should be transferred from importers/producers to each subsequent step in the food and feed supply and production chains. Moreover, for a period of five years, every operator must keep this information and be able to identify the operator from whom the product was obtained and to whom it was supplied.

The general objectives of traceability are: to facilitate GM food and feed labelling; to monitor possible potential effects on the environment and on health; and to withdraw products from the European market where unexpected adverse effects on human or animal health or the environment have been detected. Member states are obliged to set up inspection programmes including sample checks and analysis, to enforce GMO labelling and traceability regulations. This activity of Member states is regularly monitored by the European Food and Veterinary Office; and summaries of findings are made publicly available. To comply with the labelling regulations, but also for traceability and coexistence purposes there was a need of analytical tools that allow reliable identification and quantification of specific GMO events (see section 2).

4.2 CATALONIA, A PRACTICAL CASE STUDY

In view of the European regulatory framework, one of the objectives of the MARLON project was to determine the suitability of the existing documentary system for detecting intake of GM materials consumed by animals. If this was the case, this strategy could possibly be an alternative or complement to analytical methods for GMO detection.

This objective was approached by studying a particular region in Europe, Catalonia, where both GM and conventional maize varieties are commercially grown and mainly used for feed

production. Virtually all produced feed is then consumed in the same region. In addition, great amounts of maize are imported for feed production (representing about 75% consumed feed).

4.2.1 MAIZE PRODUCTION IN CATALONIA

Among EU countries, Spain cultures the highest amount of GM maize. The distribution of GM maize was not homogeneous in Spain (it corresponded to 71% in Aragón and 59% in Catalonia in 2016) or in Catalonia, where most GM maize is usually grown in two regions: Lleida and Girona (in particular the region of l'Empordà), with 59% and 64% GM maize, respectively. The production of GM and conventional maize in Catalonia during the last 13 years is represented (Figure 2). Interestingly, the post-market monitoring activities performed for this maize involved both case-specific monitoring for environmental impacts and general surveillance, the latter involving a farmer questionnaire also featuring queries on the possible impact of maize MON810 used as feed on livestock animal health (Monsanto, 2016).

Transgenic and conventional cultures cover most acreage in Catalonia (99.9%), with only 32 ha (i.e. 0.1% of maize) cultured according to organic practices. The *Consell Català de la Producció Agrària Ecològica* (CCPAE, http://www.ccpae.org/) is the official organism in Catalonia accredited to carry out the required inspections (at least once a year) to guarantee the Ecological Agricultural Production and allow the corresponding labelling when applicable.

4.2.2 MAIZE ROUTE AND DOCUMENTARY TRACEABILITY TOOLS ALONG THE FEED AND LIVESTOCK CHAIN IN CATALONIA

The maize flow from the field production to the livestock farming, including the involved stakeholders is represented in Figure 3. Every specific step in the chain is governed by specific regulations that oblige documenting various aspects of the maize for traceability purposes. Compilation of all available documents should allow full traceability of the product. The

following section addresses the specific documentation required to each specific stakeholder in the maize feed chain to the livestock farming.

<< place Figure 3 about here >>

- **Field**. About 25% maize grain used for feed production is locally cultivated in Catalonia. Farmers are obliged to present a signed Declaration (*Declaració Única Agrària*, DUN) on their cultures, mentioning the seed variety used (and thus, its conventional or MON810 character), sown area, yield and, since 2013, place of commercialization (Catalan Parliament, 2013). This DUN has to be sent to the Competent Authority, the Catalan Department of Agriculture, Livestock, Fisheries, Food and the Natural Environment (*Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural*, http://www.gencat.cat/agricultura) each season. This allows a record of conventional and GMO acreage in Catalonia.

- **Dryer facilities**. Grain transport from the field to the dryer facility is usually carried out by the same farmer using a tractor. In consequence, dryer facilities are normally chosen close to the fields, i.e. about five kilometers at maximum. A register is opened upon arrival of each tractor in which the farmer and the specific field are identified, the load is specified (in kg) and the nature of the maize grain (i.e. GM or conventional) is mentioned.

The vast majority of maize exits the dryer facilities to produce feed. In Catalonia, conventional and GM maize intended for feed uses are paid the same price (note that soybean is always GM in feed). Thus, no precautions are taken to separate GM and non-GM maize and they are usually sold together as GM maize batches. As a result the GM maize content per batch may vary considerably. If multiple GM crops would be allowed for cultivation within the region, the information on the presence and quantity of individual GM varieties per batch would be lost at this stage.

- **Transport**. Transport of maize grain and feed is systematically accompanied by a specific documentation or delivery notes. This applies to all transportation along the chain, as specified in Figure 3.

The specific documentation includes the CMR, in accordance with the Convention on the Contract for the International Carriage of Goods by Road (CMR Convention). It includes the identification number (ID, specifically identifying the document number), date, company identification and vehicle specification and registration number, the type of load and the specification GM or non-GM, weight, origin (place of charge, region, and country) and destination.

Transport of products such as processed feed require additional delivery notes specifying feed composition (specifying whether GM or conventional), nutritional composition, lot number, weight and the identification of the feed manufacturer (alfa code). But as a result of the former step, information on the GMO content and identity (in the case where multiple GMOs could be grown in the region) per batch would not be available in this stage of the feed processing chain.

- **Wholesaler carriers.** Maize trade by wholesaler carriers is performed under the same documentation basis as that provided by the seller and that required by the transport and feed manufacture.

- **Imported grain.** Importation from France, representing about 25% maize used for feed, is transported by road and is accompanied by the CMR (see above). On the other hand, about 50% total maize is imported from other European countries, Brazil and USA and is transported by boat. Spanish Ministry of Agriculture, Fisheries, Food and Environment (*Ministerio de Agricultura Pesca, Alimentación y Medio Ambiente*, http://www.magrama.gob.es/en/) is the competent

authority in the Spanish harbours, thus including those in Barcelona and Tarragona (Catalonia). Each batch is systematically accompanied with the same documentation as described for national grain and transport. However, information on the detailed nature of the GMO content (i.e. the variety/varieties involved) will only be available in the case of living modified organisms (mandatory under the Cartagena Protocol on Biodiversity), and here also there will be no information on the GMO content per batch.

- **Feed manufacture.** The feed manufacture sector is economically relevant in Catalonia, with a range of companies including small cooperatives and large companies producing both for internal uses and exportation. They all essentially follow the same documentation system. Upon entry, the batch in every truck is labelled for traceability. The information in the delivery notes and transport documents (see above) are registered in the internal database. Samples of every batch are taken for quality control and kept for three months, in accordance with HACCP. This documentation is kept as well in the internal registries.

Feed companies store grain lots in silos before processing. However, grain batches are precisely identified upon further processing. Elaborated feed is loaded on lorries for distribution. Upon departure, each lorry is accompanied by documentation stating the lot identification number, date, identification of the origin, destination and transportation companies, description of the load, including composition, GM or non-GM (but not the varieties involved or the GMO content), weight, etc.

Farmers' cooperatives mainly produce feed for local consumption. In contrast, large feed companies supply local, national and international livestock production chains.

- **Livestock farming.** Livestock farmers are obliged to keep a record on the specific feed lots, including all data included in the label and supplied by the transporter, and the dates of arrival and use. This should allow identification of the specific animals fed with every specific feed lot.

4.3 TRACEABILITY DOCUMENTARY SYSTEM AS A TOOL TO MEASURE GM FEED CONSUMPTION BY ANIMALS

On the basis of the present review of an example of the documentary system and taking into account the current regulatory framework in the EU, traceability with relation to intake or not of GM-crop-derived materials is, in principle, feasible.

As observed in this description of the traceability documentary system, information on the crop (in this case, maize), including its GM or non-GM character, is recorded all along the chain from the field to the livestock farm, but not the information on the GM crop content nor on the GM varieties involved (in the case where multiple GM crops may be grown in the region, at this point in time this is only MON810 maize). Thus, upon selection of a group of animals it should be possible to identify the specific lots of feed consumed and to trace it backwards to the maize production fields (within Spain) or region (imports), but it is not possible to detail the amount of GM crop-derived ingredients nor which specific GM variety / varieties that has/have been consumed by the group of animals. For epidemiological research it would be essential to know in detail which GM variety has been fed to what extent. From this point of view, integral traceability with relation to epidemiological research would be not feasible.

The documentation accompanying a given feed lot clearly specifies the GM character of its components, if this is the case, but not varieties involved or quantitative details on content. Thus, the sole label of a given feed lot should allow answering the question on whether a specific group of animals was fed a feed containing GM-crop-derived ingredients but not which varieties in which concentrations.

Moreover, quantification of the intake of GM ingredients by an individual animal or a given group of animals is a complex issue. As required by the EU regulations, feed lots containing GM ingredients (in this study, maize) are to be labelled as such, but the percentages of GM maize

have not to be reported and are usually not measured along the feed chain. In the present study and now that only MON810 is allowed for cultivation within Europe, a rough estimation could be envisaged by tracing the maize contained in a given feed lot to the region of origin. At least for production within Spain, data are available on the percentage of GM and conventional maize sown in any given region at any given season.

5. CONCLUSIONS

As demonstrated in a case study on GM maize in Catalonia, a documentary GMO traceability system exists, as implemented throughout the EU in response to the established regulatory framework (EU, 2003a; EU, 2003b). This system allows investigators to establish whether a specific livestock cohort was exposed or not to GM feed in general terms. More detailed information on the particular GM plant varieties and GM percentages is not available, though, and therefore the system cannot contribute to post-market monitoring of a specific GM plant.

There are numerous analytical methods suitable to detect, identify and quantify GMO-derived DNA and proteins. These methods are most suitable for use on plant raw material, although often they are suitable for analysis of processed feed (especially those based on DNA). As any protein, feed proteins derived from GMOs are mainly degraded in the animal gastro-intestinal tract. This explains why they have not been detected in animal tissues and derived products. On the contrary, dietary DNA is not completely degraded and in some cases adsorption of DNA fragments into tissues can occur. The probability of detecting GMO-related DNA in animal samples strongly depends on the type of sample, the amount of GMO in the ingested feed, or the copy number of the transgene. DNA fragments derived from the foreign DNA introduced into GMOs have been detected in faeces and GI tract but only occasionally in animal fluid and organ samples. No transgene DNA has been reported in animal-derived secondary products like meat, milk or eggs.

In consequence, direct analysis of commercial animal-derived food products does not allow, with the current knowledge, establishing whether the original animal was fed any GMO-derived feed products.

ACKNOWLEDGEMENTS

This work was carried out as part of the MARLON project ("Monitoring of Animals for Feed-related Risks in the Long Term"), financially supported by the 7th Framework Programme of the European Community for Research, Technological Development and Demonstration Activities (FP7), Grant Agreement No. 312031.

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FIGURE CAPTIONS

Figure 1. Schematic representation of the number of studies classified according to the type of sample analyzed and livestock species.

Figure 2. Production of GM and conventional maize in Catalonia from 2004 to 2016. Evolution of the acreage (ha) (A) and percentages (B) of GM and conventional maize.

Figure 3. Flowchart of maize route and documentary requirements along the production chain. T, transport.

TABLE HEADINGS

Table 1. *In silico* tools for GMO analysis. List of databases of analytical methods for GMO detection and on-line applications to support GMO analysis.

Table 2. Transfer of GM DNA to animal tissues. DNA detection in livestock and fish.

Table 3. Transfer of GM DNA to animal tissues. DNA detection in non-livestock.

SUPPLEMENTARY MATERIAL

Supplementary material S1. Literature search – selection of available data on transfer of DNA to animal tissues

SUPPLEMENTARY MATERIAL S1

Literature search – selection of available data on transfer of DNA to animal tissues

Material for the literature review was selected from a reference lists pertaining to "Transgenic DNA and livestock products" (updated April 2014) created by the Federation of Animal Science Societies (FASS) and from searches carried out in bibliographic databases such as BIOSIS, CABA, EMBASE, FROSTI, FSTA, MEDLINE, PUBMED and SCIENCE DIRECT using various combinations of key words including "analysis", "animal", "blood", "broiler", "cattle", "dairy cow", "detection", "DNA", "fate", "feed", "foreign DNA", "genetically modified organism", "GIT", "GM" "goat", "livestock", "milk", "poultry", "PCR", "sheep", "test", "tissue", "traceability" and "transfer. A broad research was initially performed and articles out of the scope were subsequently eliminated Relevant papers were selected based upon evaluation of the abstracts as well as from the reference lists of extracted papers; in total, 83 studies specifically focusing on detection of foreign DNA in animals, were selected for review.

TABLES

Table 1. *In silico* tools for GMO analysis. List of databases of analytical methods for GMO detection and on-line applications to support GMO analysis.

DATABASE	DESCRIPTION	REFERENCE
GMDD: GMO Detection Method Database	Provides detailed information on DNA- and protein-based methods for GMO analysis	(Dong et al., 2008)
The CropLife International Detection Methods Database	Provides detailed information on DNA- and protein-based methods for GMO analysis	Initiative of Croplife International
GMOMETHODS: The European Union Database of Reference Methods for GMO Analysis	Provides reliable and harmonized information on EU reference methods (validated PCR-based methods) for detection of genetically modified organisms (GMOs)	(Bonfini et al., 2012)
EUginius: EUropean GMO INItiative for a Unified database System	Analysis tool based on the GMO elements previously identified and those that have been found negative in the screening. Provides accurate information of major and relevant issues regarding the presence, detection and identification of GMOs with a focus on the situation in the European Union as well as world-wide coverage. It includes sections of "GMO selection", "Detection Methods", "GMO analysis tool", "GMO authorisation search" and "Search for GMO"	Initiative of the Federal Office of Consumer Protection and Food Safety (Berlin, DE) and RIKILT Wageningen UR (Wageningen, NL)

GMO Screening Table	Provides a GMO method matrix based on a specific set of methods targeting the most regularly present genetic elements and constructs. All methods are validated and included in GMOMETHODS database. Excel document available at http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/screening_tabelle_gvoNachweis.xls?__blob=publicationFile	(Waiblinger et al., 2010)
Extended Element Screening Approach	Provides a GMO method matrix based on 15 TaqMan real-time PCR methods (verified against a large set of reference materials). This extended screening approach reduces the number of subsequent analyses, mainly for feed samples, as well gives indications for unauthorized GMOs	(Scholtens et al., 2013)
GMOseek matrix	Comprehensive online open-access tabulated database which provides a reliable, comprehensive and user-friendly overview of GMO events and different genetic elements. It assists in selecting the targets for a screening analysis, interpreting the screening results, checking the occurrence of a screening element in a group of selected GMOs, identifying gaps in the available pool of GMO detection methods, and designing a decision tree	(Block et al., 2013)
JRC GMO-matrix	Platform that links GMO sequence information found in the Central Core DNA Sequence Information System (CCSIS) and information (primers and probes sequences) existing in the GMOMETHODS database. The application perform in silico simulations of PCR amplification and, when applicable, probe binding using bioinformatics tools, such as re-PCR and matcher	(Angers-Lousteau et al., 2014)
JRC GMO-Amplicons	Nucleotide database of putative GMO-related sequences, obtained by PCR simulation screening of public nucleotide sequence databanks, including patents and available whole plant genomes	(Petrillo et al., 2015)

GMO*finder*	Database for collection and interpretation of information related to the screening for genetically modified organisms. Available on request from the corresponding author (sven.pecoraro@lgl.bayern.de)	(Gerdes et al., 2012)
CoSYPS: Combinatory SYBR Green qPCR Screening	Decision support system based on the SYBR® Green qPCR analysis method to screen the unknown samples for the presence of common genetic elements such as promoters, terminators, coding or other sequences which are present in different Genetically Modified (GM)-events. The system is modular and any method can be added at any time when necessary	(van den Bulcke et al., 2010)
GMO Checker	Provides a screening application for the complete and semi-quantitative detection of genetically modified crops. It is a combination of 14 event-specific and 10 element specific methods. An Excel spreadsheet application can be downloaded from the internet (http://cse.naro.affrc.go.jp/jmano/UnapprovedGMOChecker_v2_01.zip)	(Mano et al., 2009)

TABLES

Table 2 - Transfer of GM DNA to animal tissues. DNA detection in livestock and fish.

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	Plant reference gene	Positive tissue
Cattle							
Klotz and Einspanier, 1998	feeding glyphosate tolerant soya	cow	blood, milk	No		Yes (199 bp, *cp* gene)	blood
Einspanier et al., 2001	feeding Bt maize	cow (beef and dairy)	blood, feces, intestinal content, milk, muscle, liver, spleen	Yes (189 bp)	duodenal content	Yes (199 and 532 bp, *cp*)	blood, duodenal content, lymphocytes milk
Phipps et al., 2002	feeding glyphosate tolerant soya	cow (dairy)	milk	No		nt	
Poms et al. 2003	Feeding soybean maize	cow (dairy)	blood, feces, milk	nt		No	
Calsamiglia et al., 2003	feeding Bt and glyphosate tolerant maize	cow (dairy)	milk	No		nt	
Jennings et al., 2003c	feeding Bt cottonseed, Bt maize	cow (beef and dairy)	kidney, liver, milk, spleen	No		No	
Phipps et al., 2003	feeding Bt maize, glyphosate tolerant soya	cow (dairy)	blood, feces, intestinal and rumen content, milk	Yes (203 bp)	Solid phase of rumen and intestinal content	Yes (189 and 351 bp, *cp*)	all samples

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Yonemochi, et al., 2003	feeding Bt maize	cow (dairy)	blood, GIT heart, kidney, lung, milk, pancreas	No		nt	
Castillo et al., 2004	feeding Bt cottonseed glyphosate tolerant cottonseed	cow (dairy)	milk	No		No	
Chowdhury, et al., 2004	feeding Bt maize	cow (calf)	blood, GIT content, kidney, liver, lymph node, muscle, spleen	Yes (110 bp)	entire GIT content	Yes (231 bp, *cp*)	entire GIT content, kidney, liver, lymph node, muscle spleen
Einspanier et al., 2004	Feeding Bt maize	cow	blood, feces, GIT plus content	No		Yes (199 bp, *cp*)	entire GIT plus content
Nemeth et al., 2004	feeding Bt maize	cow (beef and dairy),	milk, muscle	No		Yes (173 bp, *cp*)	milk
Agodi et al., 2006	supermarket (milk) Bt maize, glyphosate tolerant soya	cow (milk)	milk	Yes (possible contamination)	milk	Yes (69 bp, *zein*; 80 bp, *lectin*)	milk
Phipps et al., 2005	feeding glufosinate tolerant maize	cow (dairy)	milk	No		No	

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Calsamiglia et al., 2007	feeding Bt maize	cow (dairy)	milk	No		nt	
Bertheau et al., 2009	feeding Bt maize	cow (dairy)	blood	No		Yes (170 bp, *adh*; 152 bp, *26S rRNA*;118 bp, *psaB*)	blood
Guertler et al., 2009	feeding Bt maize	cow (dairy)	milk	No		nt	
Guertler et al., 2010	feeding Bt maize	cow (dairy)	blood, feces, milk, urine	Yes (206 bp)	feces	nt	
Sieradzki et al. 2013	feeding Bt maize, glyphosate tolerant soya	calves	Blood, liver, spleen, kidney, pancreas, muscle, stomach, duodenum, jejunum, colon, excreta,	Yes (123 bp, *CaMV 35S*; 118 bp, *nos*)	stomach	Yes (226 bp, *invertase*; 118 bp, *lectin*)	stomach, duodenum
Furgał-Dierżuk et al., 2015	feeding Bt maize, glyphosate tolerant soya	cow	milk, rumen after 8 h	No		No	

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
De Giacomo et al., 2016	real life husbandry Bt maize, glyphosate tolerant soya	cow	milk,	No		No	
Sheep							
Duggan et al., 2003	feeding Bt maize pipette (oral cavity) plasmid DNA	sheep	feces, oral cavity, rumen content	Yes (1914 bp and 211 bp, *cry1a*; 350 bp and 600 bp, *bla*)	rumen content (Bt maize)	Yes (226 bp, *invertase*; 370 bp, *16S rRNA*)	oral cavity (plasmid DNA), rumen content
Alexander, et al., 2006	feeding glyphosate tolerant rapeseed	sheep	blood, duodenal and rumen content, feces	Yes (108 bp - 1363 bp)	duodenal and rumen content	nt	
Sharma et al., 2006	feeding glyphosate tolerant canola	sheep (lamb)	GIT and GIT content, kidney, liver, spleen	Yes (179 bp - 527 bp)	entire GIT and GIT content apart from esophagus	Yes (540 bp, *rubisco LF*; 186 bp, *rubisco SF*; 520 bp, *cp*)	entire GIT and GIT content, all tissues
Trabalza-Marinucci et al., 2008	feeding Bt maize	sheep	GIT, liver, lymph nodes, rumen content	No		nt	

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Goat							
Rizzi et al., 2008	feeding Bt maize	goat	milk	No		Yes (532 bp and 199 bp, *cp*)	milk
Rabbit							
Tudisco et al., 2010	Feeding barley soybean	rabbit	blood, liver, kidney, spleen, muscle tissue and digesta	nt		Yes (100 bp, *cp*)	blood, liver, kidney, spleen, muscle tissue and digesta
Morera et al., 2016	feeding glyphosate tolerant soya	rabbit	blood, liver, kidney, heart, stomach, jejunum, muscle and adipose tissue	Yes (84 bp)	stomach digesta and feces	Yes (74 bp, *lectin*)	stomach digesta and feces
Deer							
Guertler et al., 2008	feeding	deer	GIT content, kidney, liver, lymph nodes, muscle, spleen	No		Yes (173 bp, *rubisco*; 329 bp, *zein*)	entire GIT content, liver, lymph nodes, muscle, spleen
Pig							
Weber and Richert, 2001	feeding Bt maize	pig	muscle	No		No	

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Chowdhury et al., 2003a	feeding Bt maize	pig	blood, GIT content	Yes (110 bp and 437 bp)	cecal, duodenal, ileal, rectal, stomach content	Yes (226 bp, *invertase*; 242 bp, *zein*; 1,028 bp, *ribulose*)	entire GIT content
Chowdhury et al., 2003b	feeding Bt maize	pig	GIT content	Yes (103 and 170 bp)	cecal and rectal content	Yes (242 and 329 bp, *zein*)	cecal and rectal content
Jennings et al., 2003b	feeding glyphosate tolerant soya	pig	muscle	No		No	
Reuter and Aulrich, 2003	feeding Bt maize	pig	blood, GIT content, kidney, liver, lymph nodes, muscle spleen,	Yes (211 bp)	rectal content	Yes (140 bp, *cp*)	blood, all organs and tissues
Nemeth et al., 2004	feeding Bt maize	pig	muscle	No		Yes (173 bp and 500 bp, *cp*)	muscle
Broll et al., 2005	feeding Inulin potato silage	pig	blood, GIT, kidney, liver, muscle, spleen, stomach, thymus	Yes (104 bp)	stomach	Yes (106 bp, *pat*)	intestine

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Mazza et al., 2005	feeding Bt maize	pig (piglet)	blood, kidney, liver, muscle, spleen	Yes (519 bp)	blood, kidney, liver, spleen	Yes (439 bp, *zein*; 532 bp, *sh2*)	blood, kidney, liver, spleen
Beagle et al., 2006	feeding glutamate-dehydrogenase maize	pig	GIT content, liver, muscle	Yes (419 bp)	ileal and stomach content	nt	
Sharma et al., 2006	feeding glyphosate tolerant canola	pig,	GIT content, and GIT tissues kidney, liver, spleen	Yes (179-527 bp)	entire GIT content, and GIT tissues	Yes (520 bp, *cp*; 186 bp and 540 bp; *rubisco*)	entire GI tract content, all tissues
Yonemochi, et al., 2010	feeding Bt maize	pig	blood, liver, muscle	No		nt	
Swiatkiewicz et al., 2011	feeding Bt maize, glyphosate tolerant soya	pigs	stomach, duodenum, ileum, caecum colon, blood, liver, spleen, lung, muscle.	Yes (172 bp and 170 bp)	stomach, duodenum	nt	Stomach, duodenum
Walsh et al., 2011	feeding Bt maize	pig	blood, GIT content, heart, kidney, liver, muscle, spleen	Yes (149 bp)	GIT content	Yes (213 bp, *Sh2*)	GIT content

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Walsh et al., 2012	Feeding (long term-110days) Bt maize	pig	blood, GIT content, heart, kidney, liver, muscle, spleen	Yes (149 bp and 211 bp)	stomach	Yes (173 bp and 226 bp, *rubisco*)	blood, GIT content, heart, kidney, liver, muscle, spleen
Buzoianu et al. 2012	feeding Bt maize	Pig: sows during gestation, lactation and offsprings	blood and faeces	No		Yes (173 bp, *rubisco*)	blood and faeces
			blood and tissues	No		Yes (173 bp, *rubisco*)	blood and muscle
Swiatkiewicz et al., 2013	feeding Bt maize, glyphosate tolerant soya	pigs	blood	No		No	
Sieradzki et al. 2013	feeding Bt maize, glyphosate tolerant soya	pig	Blood, liver, spleen, , muscle, stomach, duodenum, jejunum, excreta,	Yes (123 bp and 118 bp)	stomach, duodenum	Yes (226 bp, *invertase*; 118 bp, *lectin*)	stomach, duodenum
Poultry							
Einspanier et al., 2001	feeding Bt maize	chicken (broiler and layer	blood, egg, feces , intestinal content, milk, muscle, liver, spleen	No		Yes (199 bp, *cp*)	kidney, liver, muscle, spleen

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Klotz et al., 2002	feeding (pig) Bt maize supermarket (chicken)	chicken (broiler),	blood, GIT plus content, liver, muscle, lymph nodes, spleen	No		Yes (199 bp, *cp*)	muscle, stomach
Yonemochi et al. 2002	feeding Bt maize	chicken (broiler chicks)	blood, liver, muscle	No		nt	
Jennings et al., 2003a	feeding Bt maize	chicken (broiler)	kidney, liver, muscle, spleen	No		No	
Tony et al., 2003	feeding Bt maize	chicken (broiler)	blood, heart, GIT, kidney, liver, muscle, spleen, thymus	Yes (129 bp)	blood, liver, muscle, spleen	Yes (199 bp and 79 bp, *cp*)	blood, cecum, crop, duodenum, gizzard, ileum, liver, muscle, proventriculus, rectum, spleen
Marzok, 2004	feeding Bt maize	chicken (broiler)	blood, GIT content, heart, kidney, liver, muscle spleen, thymus	No		Yes (199 bp, *cp*)	blood, kidney, liver, muscle, spleen
Nemeth et al., 2004	feeding Bt maize	chicken,	muscle	No		Yes (73 bp, *cp*)	muscle
Aeschbacher et al., 2005	feeding Bt maize	chicken (broiler and layer)	blood, GIT, liver, muscle, spleen	No		Yes (226 bp, *invertase*)	blood, crop, gizzard, small intestine, liver, muscle, spleen

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Deaville and Maddison, 2005	feeding Bt maize, glyphsophate tolerant soya	chicken (broiler)	blood, bursa, GIT content, gizzard, heart, kidney, liver, muscle, spleen	Yes (203 bp, *cry*; 171 bp, *cp4epsps*; 195 bp, *CaMV35*)	gizzard, small and large intestinal content	Yes (167 bp, *rubisco*; 240 bp, *lectin*; 240 bp, *hmp*)	blood, duodenal , gizzard, small and large intestinal content, gizzard, kidney, liver, muscle, spleen,
Flachowsky et al., 2005	feeding Bt maize	quail	egg, GIT, heart, kidney, liver, muscle, spleen	Yes	entire GIT	nt	
Rossi et al., 2005	feeding Bt maize	chicken (broiler)	blood, GIT content	Yes (1,815 bp)	, gizzard, content	Yes (439 bp, *zein*; 1,830 bp, *Sh2*)	blood, cecal, crop, gizzard, jejunal content zeingizzard content SH2
Rehout et al., 2008a	feeding glyphosate tolerant soya	chicken (broiler)	blood	Yes (128 bp)	blood	Yes (110 bp, *lectin*)	bloodr
Rehout et al., 2008b	feeding Bt maize, glyphosate tolerant soya	chicken (broiler)	kidney, liver	Yes (128 bp)	liver	Yes (110 bp, *lectin*)	liver
Swiatkiewicz et al., 2010	feeding Bt maize, glyphosate tolerant soya	chicken (broiler)	Gizzard, duodenum, jejunum, ileum, caecum, blood, liver, spleen, breast muscle.	Yes (172 bp and 170 bp)	gizzard	Yes (118 bp, *lectin*; 226 bp, *invertase*)	gizzard

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Ma et al., 2013	Feeding Phytase transgenic corn	laying hens	digestive tract , blood, heart, liver, spleen, kidney, breast muscle, and eggs	Yes (678 bp)	gizzard	Yes (226 bp, *invertase*)	Digestive tract
Korwin-Kossakowska et al., 2013	Feeding (four generations) Bt maize, glyphosate tolerant soya	Japanese quail	Breast muscle, gizzard, liver, spleen, duodenum, kidney and heart, and eggs	No		No	
Sieradzki et al., 2013	feeding Bt maize, glyphosate tolerant soya	Broiler and laying hens	Blood, liver, spleen, breast muscle, gizzard, duodenum, Jejunum, ileum, caecum, cloaca, excreta, eggs	Yes (123 bp, *CaMV35S*; 118 bp, *nos*)	gizzard, duodenum,	Yes (226 bp, *invertase*; 118 bp *lectin*)	gizzard, duodenum,
Fish							
Sanden et al., 2004	feeding glyphosate tolerant soya	fish (salmon)	brain, GIT content, liver, muscle	Yes (125 and 195 pb)	mid and distal intestinal content, pyloric content, stomach	No	
Nielsen et al., 2005	feeding (DNA spike) Bt maize, glyphosate tolerant soya	fish (salmon)	blood, GIT content, kidney, liver	Yes (up to 81 bp, 84 bp and 151 bp)	blood, GIT content, kidney, liver	nt	
Chainark et al., 2006	feeding defatted soya	fish	muscle	Yes (220 bp)	muscle	No	

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Chainark et al., 2008	feeding glyphosate tolerant soya	fish (trout)	blood, brain, GI tract contents, kidney, liver, muscle, spleen	Yes (220 bp)	entire GI tract content, kidney, leucocytes, muscle	Yes (257 bp, *cp*)	entire GI tract content, leucocytes, spleen, stomach
Sanden et al., 2011	feeding glyphosate tolerant soya	fish (salmon)	GIT	No		Yes (180 bp, *lectin*)	entire GIT

nt = not tested

cp = chloroplast gene

TABLES

Table 3. Transfer of GM DNA to animal tissues. DNA detection in non-livestock.

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	Plant reference gene	Positive tissue
Schubbert et al., 1994	feeding phage M13 DNA	mouse	blood, feces		transient survival M13 DNA in blood and feces		blood
Schubbert et al., 1997	feeding phage M13 DNA	mouse (pregnant)	blood, feces, GIT, kidney, liver, spleen	-	transient M13 DNA: blood, gut and feces		kidney, leucocyte, liver
Schubbert et al., 1998	feeding phage M13 DNA, plasmid DNA	mouse (pregnant)	fetus	nt		Yes	across placenta to fetus
Hohlweg and Doerfler, 2001	feeding, injection (intramuscular) soya, plasmid DNA, GFP (green fluorescent protein)	mouse	feces, GIT, liver, spleen	*gfp*		Yes (*rubisco*)	git,feces, liver, spleen
Palka-Santini et al., 2003	feeding, injection (GIT) GFP and M13 DNA	mouse	GIT, kidney, liver, spleen		GIT		GIT, kidney, liver, spleen

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Netherwood et al., 2004	feeding glyphosate tolerant soya	human	feces, ileum content, large intestine	Yes (180 bp)	ileum content (ileostomy subjects)	*Le1*	ileum content (ileostomy subjects)
Zhu et al., 2004	feeding glyphosate tolerant soya	rat	masseter muscle	No		No	
Baranowski et al., 2006	feeding GM triticale	mouse	blood, kidney, liver, muscle, spleen	No		nt	
El-Sanhoty, 2006	feeding GM potato	rat	feces, GIT content, kidney, liver, lung, muscle, skin, spleen, testes	Yes (122 bp, 504 bp and 1000 bp)	entire GIT content, feces	Yes (550 bp *leucine*)	entire GIT content, feces, kidney, liver, muscle, spleen
Wiedemann et al., 2009	feeding Bt maize	wild boar	blood, GIT content, heart, kidney, liver, lung, lymph node, spleen	Yes (727 bp)	git content	Yes (173 bp, *rubisco*)	entire GIT

Authors	Type of Study	Species	Tissues sampled	Detection of DNA			
				Transgene	Positive tissue	plant reference gene	Positive tissue
Sissener et al., 2010	feeding Bt maize, glyphosate tolerant soya	zebrafish	brain, GIT, liver, muscle	Yes (92 bp)	GIT,liver	Yes (*rubisco*)	all tissues

nt = not tested

Figure 1

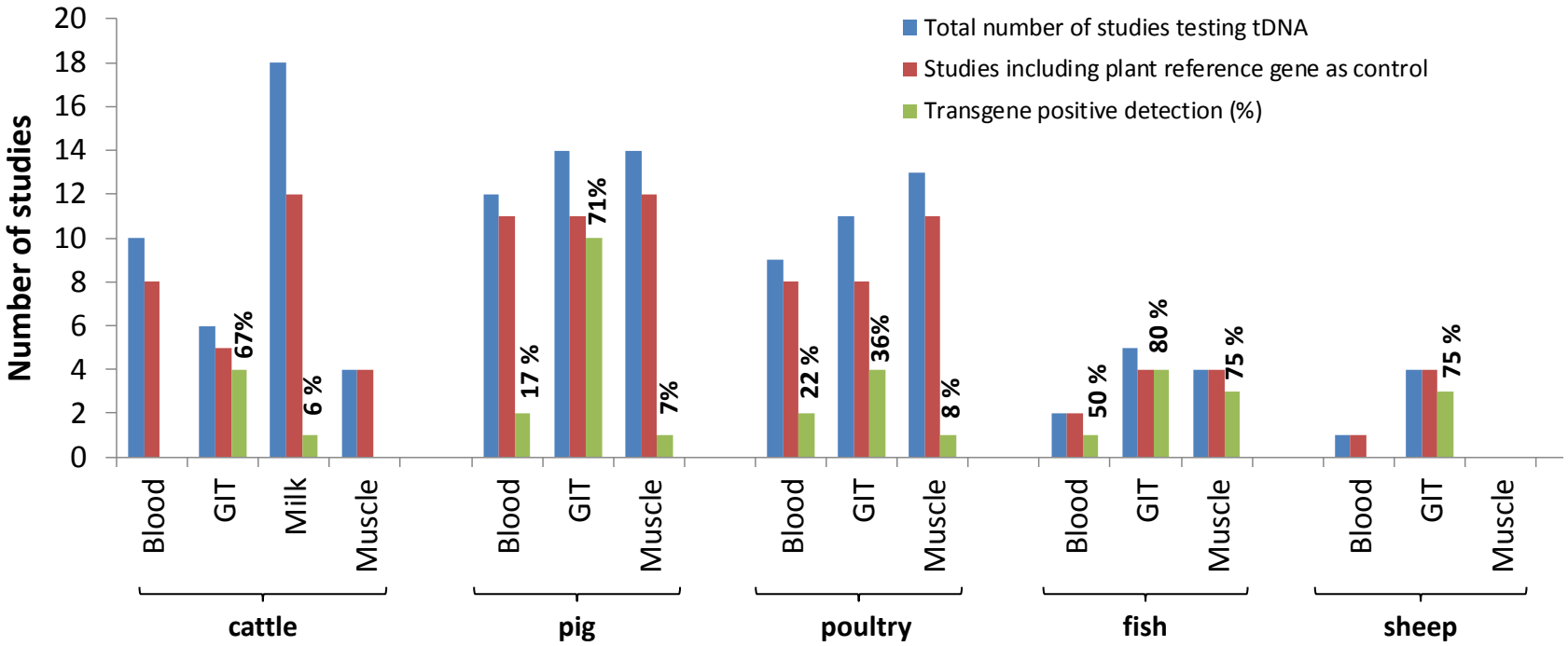

Number of studies
20
18
16
14
12
10
8
6
4
2
0
Total number of studies testing tDNA
Studies including plant reference gene as control
Transgene positive detection (%)
67%
6 %
17 %
71%
7%
22 %
36%
8 %
50 %
80 %
75 %
75 %
Blood
GIT
Milk
Muscle
cattle
pig
poultry
fish
sheep

Figure 2

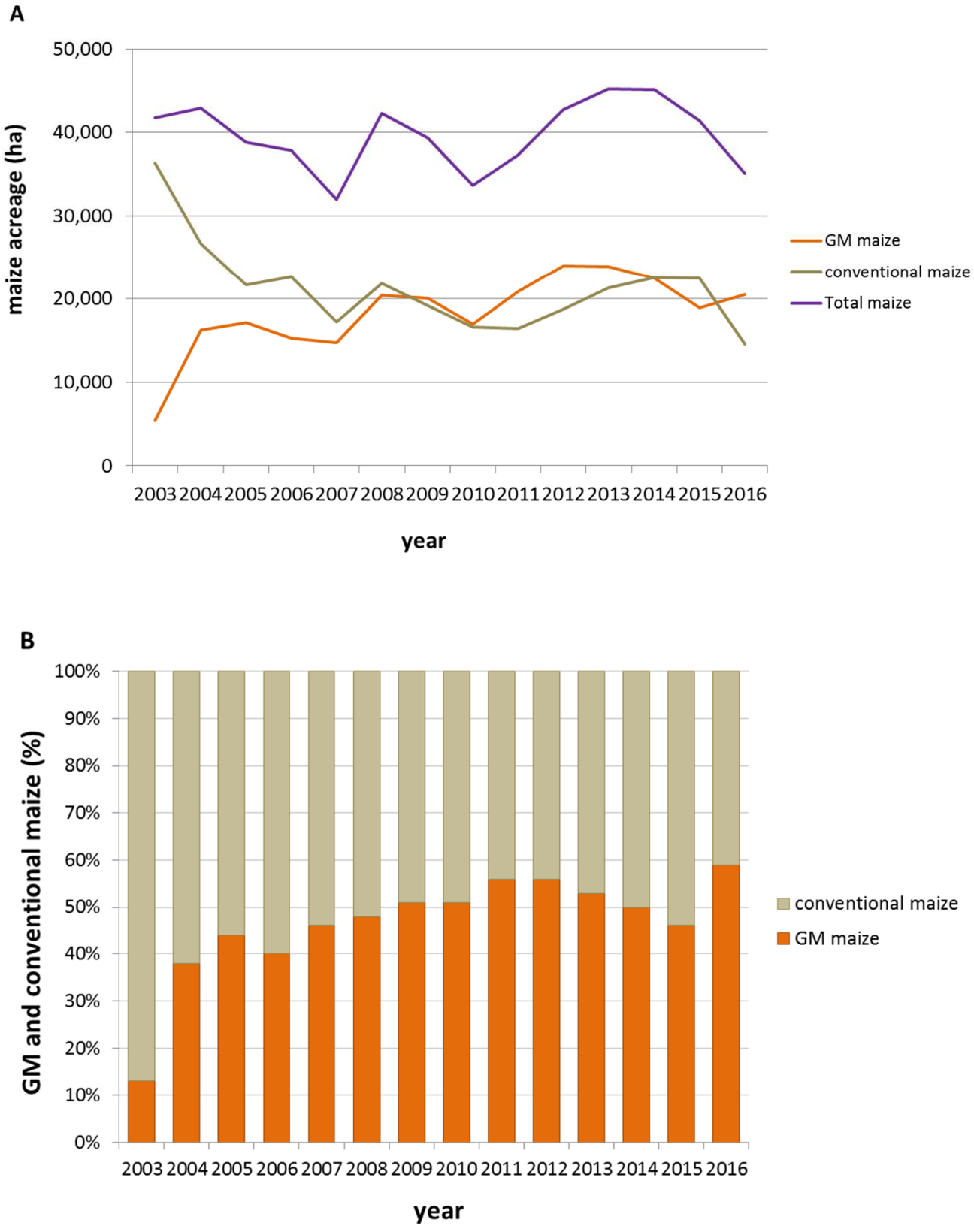
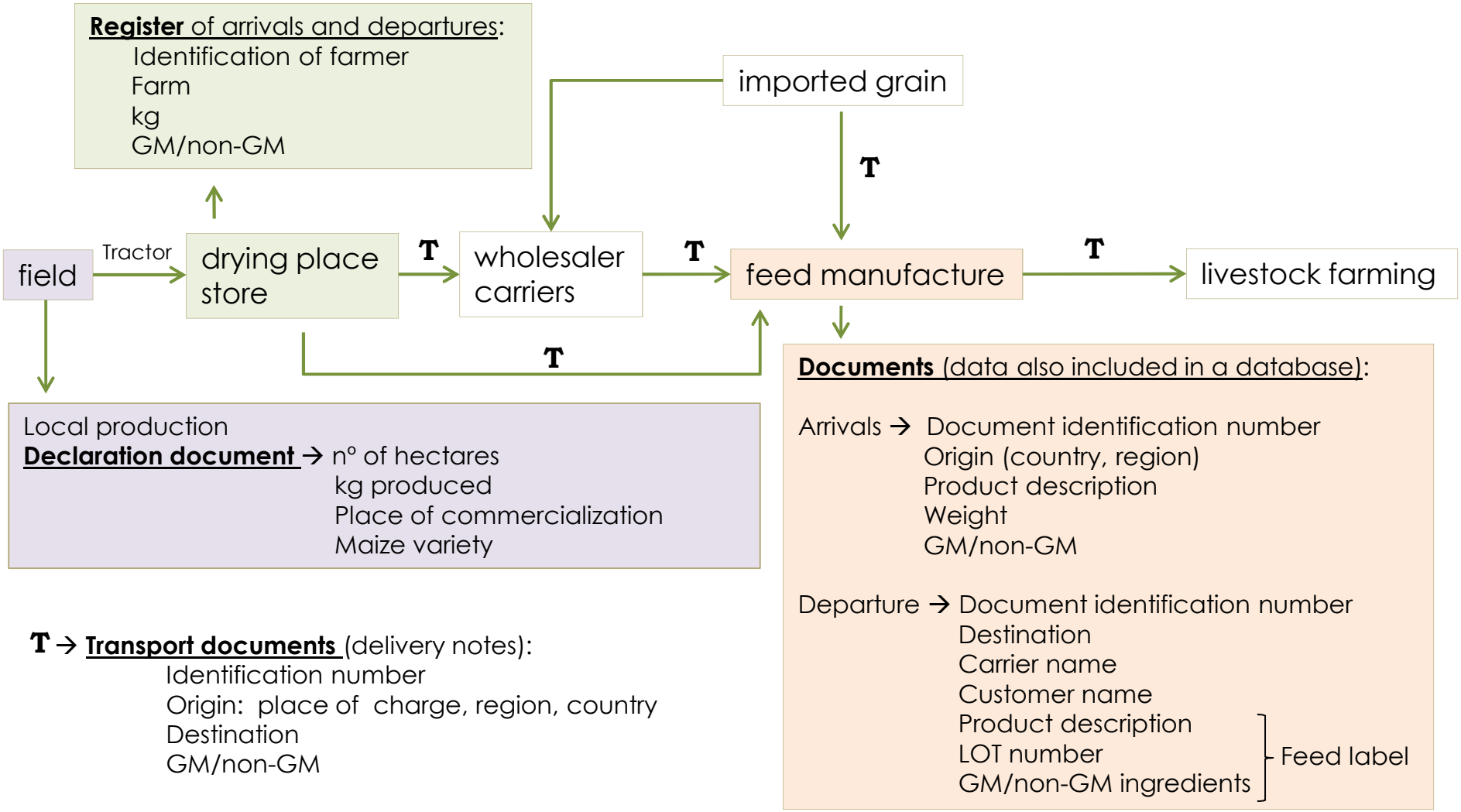

A
maize acreage (ha)
50,000
40,000
30,000
20,000
10,000
0
2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016
year
GM maize
conventional maize
Total maize
B
GM and conventional maize (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
2003 2004 2005 2006 2007 2008 2009 2010 2011 2012 2013 2014 2015 2016
year
conventional maize
GM maize

Figure 3


Register of arrivals and departures:
Identification of farmer
Farm
kg
GM/non-GM
imported grain
T
field
Tractor
drying place store
T
wholesaler carriers
T
feed manufacture
T
livestock farming
T
Local production
Declaration document → n° of hectares
kg produced
Place of commercialization
Maize variety
Documents (data also included in a database):
Arrivals → Document identification number
Origin (country, region)
Product description
Weight
GM/non-GM
Departure → Document identification number
Destination
Carrier name
Customer name
Product description
LOT number
GM/non-GM ingredients
Feed label
T → Transport documents (delivery notes):
Identification number
Origin: place of charge, region, country
Destination
GM/non-GM

RESEARCH HIGHLIGHTS

- Reviewed data show that both DNA- and protein-based methods can be used to detect genetically modified ingredients in feed
- Transfer of transgenic DNA/proteins from consumed feed to animal tissues is not a good biomarker for GM exposure measurement
- Traceability documentation for GM crops in the EU allow for identifying animals having consumed GM feed ingredients to a limited extent
- Traceability documentation is not able to quantify the intake and identify the specific genetically modified crops involved