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A review of the detection and fate of novel plant molecules derived from biotechnology in livestock production

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Abstract

Since the commercialization of the first genetically modified (GM) crop in 1996, the amount of arable land dedicated to the production of GM feed has increased significantly. Despite widespread adoption of GM foods and feeds, public perception of their safety remains mixed. To provide consumers the opportunity for choice, some countries have adopted mandatory labeling of GM foods and feeds when their adventitious presence exceeds a defined threshold percentage. Methods for detecting and quantifying GM plants in feeds include protein- and DNA-based assays, but their sensitivity may be influenced by the techniques used in feed processing. Interest in the consumption of transgenic protein and DNA has prompted investigations of their fate within the gastrointestinal tract of livestock

Abbreviations: Ab, antibodies; ARM, antibiotic resistant marker; CaMV, cauliflower mosaic virus; CRMs, certified reference materials; CTAB, hexadecyltrimethylammonium bromide; ELISA, enzyme-linked immunosorbent assay; FAO, Food and Agriculture Organization; fwt, fresh weight tissue; GIT, gastrointestinal tract; GM, genetically modified; IRMM, Institute for Reference Materials and Measurements; LOD, limit of detection; LOQ, limit of quantification; OECD, Organisation for Economic Co-operation and Development; QC-PCR, quantitative competitive PCR; RT-PCR, real-time PCR; RFLP, restriction fragment length polymorphism; $R.S.D._r$, repeatability; $R.S.D._R$, reproducibility; WHO, World Health Organization

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and the potential to which transgenes or their products may be incorporated into tissues. Transgenic protein has not been detected in any animal tissues or products. Fragments of DNA from endogenous, high-copy number chloroplast genes from plants have been detected in poultry, pig and ruminant tissues. Low-copy endogenous and transgenic DNA in animal tissues have been detected but to a lesser extent than high-copy genes. Current research suggests that the passage of dietary DNA fragments across the intestinal wall is a natural physiological event, the likelihood of which is dependent on their concentration in the feed. To date, the transgenic traits approved for expression in crops used as feeds have not posed a safety concern for livestock.

Keywords: Genetically modified plant; Genetically modified feed; Transgene detection; Gene transfer; Recombinant protein detection; Digestion

1. Introduction

Advances in molecular biology and recombinant DNA techniques have made it possible to engineer plant genomes. Compared with traditional plant breeding methods, such as artificial crossing or hybridization, biotechnology now allows for the introduction of DNA from outside the plant kingdom. Selective inclusion of single or multiple traits can be performed to change the quality of agricultural crops (Gassen and Hammes, 2001). The majority of genetically modified (GM) crops currently produced have been engineered to enhance agronomic performance by transformation with genes encoding herbicide tolerance or pest resistance (James, 2004). However, the potential of GM plants is not limited to agronomic enhancement but may also serve as a means of enhancing the nutritional status of feed for livestock or food for humans (Matissek, 1998). In 1996, the first GM varieties of major crops used as feedstuffs for livestock entered the market in North America. These included herbicide-tolerant soybeans and canola and pest-protected maize and cotton. During the 9-year period 1996–2004, the global area of GM crops increased over 47-fold. In 2004, GM crops were grown on a total of 81.0 million ha world wide (James, 2004). Regulations concerning GM plants were established by major international organizations prior to their commercialization. The policy of requiring establishment of substantial equivalence was first introduced by the Organisation for Economic Co-operation and Development (OECD, 1993) and was adopted by both the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as the most appropriate method to ensure the safety of GM plants (FAO/WHO, 2000). Testing for substantial equivalence provides a framework for the safety assessment by comparing similarities and differences between a biotechnology-derived plant and an appropriate counterpart such as the parental line from which the GM plant was derived. Once certain factors of the plants have been determined to be equivalent, the differences, which may only relate to the novel transgenic trait, become the focus of the safety assessment. Detailed studies of the feeding qualities of GM plants for livestock and their nutritional evaluation have been reviewed previously (Flachowsky et al., 2005; Aumaitre et al., 2002; Flachowsky and Aulrich, 2001).

Throughout the world, controversy remains both at public and at scientific levels about the benefits and risks regarding GM crops entering the food- and feed chains as well as regulations presently in place (Finucane, 2002; Shelton et al., 2002). Between 1996 and

1999, Europeans became increasingly opposed to GM foods (Gaskell et al., 2000). A more recent study showed that European consumers place significantly greater value on beef from cattle fed conventional maize grain as compared to those fed genetically modified maize (Lusk et al., 2003).

In particular, there has been interest in the stability and presence of recombinant DNA or proteins expressed by that DNA entering the food chain. Given that livestock consume large amounts of plant material and that high-protein feeds are among the most common GM crops, there is considerable opportunity for livestock to consume significant quantities of recombinant DNA and protein. Reliable detection and quantification of transgenic traits in livestock feed is required if legislated allowable levels of transgenic feed in import shipments are to be honored for countries with labeling legislation. The objective of the present paper is to review the methods used to detect transgenic feeds in livestock diets and the fate of recombinant proteins and DNA consumed by livestock.

2. Detection of GM plants

2.1. Rationale for detecting GM feeds

Monitoring the presence of GM plants in a wide variety of food and feed matrices is important to countries with labeling laws for approved GM varieties. In addition, countries may want to test for unapproved GM varieties. In the United States, which does not require labeling of GM products, two recent events with implications for human health have emphasized the importance of being able to detect GM foods and feeds. The first incident occurred in 2000 with the detection in human food of a GM maize that was only approved for use in animal feed (Dorey, 2000). The second was the accidental shipment of an unapproved GM maize variety for cultivation between 2001 and 2004 (Herrera, 2005).

Labeling regulations for GM foods have been implemented by various countries. Recently, the European Union (EU) has extended regulations concerning GM foods to include animal feeds and feed additives. According to Regulation (EC) No. 1829/2003 (EC, 2003a), all foods and feeds containing or derived from approved GM products in amounts greater than a 0.9% threshold are subject to labeling rules. In addition, a 0.5% labeling threshold has been mandated for GM crops that have been given a favorable risk assessment but are not yet approved within the EU. Unapproved varieties are managed with zero tolerance. This differs from previous legislation by which foods only had to be labeled if GM plant material, namely recombinant DNA or proteins, could be detected above the threshold (EC, 2000). The thresholds account for the adventitious or technically unavoidable presence of GM organisms in foods/feeds. Labeling of feeds containing GM ingredients informs farmers and gives them the choice of using such feed for their livestock. However, products such as milk, meat, and eggs, that are derived from livestock fed transgenic feeds are exempt from EU-labeling laws. Currently, only the EU and Switzerland have labeling regulations pertaining to GM feed (ISAAA, 2005). Other countries with mandatory or voluntary labeling legislations for GM foods are reported in Table 1.

In compliance with Regulation (EC) No. 1830/2003 (EC, 2003b) and in order to help implement the labeling legislation of Regulation (EC) No. 1829/2003, any feed product

Table 1
Regulations concerning the labeling of GM food or feed products in select countries[a]

Country	Labeling status	Threshold (percent)	Date of implementing threshold regulation
Australia and New Zealand	Mandatory	1	December 2001
Brazil	Mandatory	4	December 2001
Canada	Voluntary[b]	–	November 1994
China	Mandatory	0	July 2001
Czech Republic	Mandatory	1	Not available
European Union	Mandatory[c]	0.9, 0.5 food and feed	July 2003
Hong Kong	Voluntary	5	February 2001
Israel	Mandatory[d]	1	Not available
Japan	Mandatory for selected products	5	April 2001
Korea	Mandatory for selected products[d]	3	13 June 2001
Malaysia	Mandatory	3	Proposal
Russia	Mandatory for selected products	5	1 September 2002
Switzerland	Mandatory	2 or 3 (feed[e]), 0.5 (imported seeds)	Not available
Taiwan	Mandatory	5	Proposal
Thailand	Mandatory for selected products	5	Proposal
United States	Voluntary		January 2001

Data have been converted to SI units.

[a] Data from Jia (2003) and ISAAA (2005).

[b] Labeling required if safety concerns (allergenic, change in nutritional composition) exist.

[c] Labeling required at a 0.9% threshold for approved GM organisms or 0.5% for GM organisms given a favorable risk assessment but not yet approved. Includes both feed and food products.

[d] Labeling required only if recombinant DNA or proteins are detected.

[e] Threshold for feeds containing raw material of a single source is 3%. For mixed feeds, a threshold of 2% exists.

containing more than the allowable thresholds of GM plant content must be accompanied by proper documentation stating that it consists of GM constituents within the EU. An unique identifier describing the GM crop must be transmitted at each processing stage prior to the marketing of the food or feed product. However, for these maximum allowable levels to be imposed, validated analytical tests are required to quantify the level of the transgenic trait in mixed feeds. The required method to detect GM plants in the EU is not described but protocols for detecting and quantifying GM material are available from the Joint Research Centre, a Directorate General within the European Commission. Both the method of sampling and the precision of the analytical method affect the accuracy and precision of detecting transgenic traits in feed (Heinemann et al., 2004). As production of GM plants involves introducing transgenic DNA that either encodes a protein to be expressed or is present in the antisense direction to impede protein expression, transgenic feeds can be detected by assaying either transgenic DNA or recombinant proteins.

2.2. Sampling of plant material

Proper sampling procedures are critical to reliable detection of GM products in feeds. A significant amount of error can arise from collection methods and that error increases as the concentration of GM feed decreases (Gilbert, 1999). When designing guidelines, it is important to be aware of the buyer's and seller's risks, both which decrease as the sample size increases (Whitaker et al., 2001). By chance, a shipment of seeds that contains a greater percentage of GM material than the buyer's threshold could be accepted. Conversely, a shipment that actually contains less GM material than the threshold could be rejected. For a detection methodology to be adopted, the sampling strategy and analysis method should achieve an acceptable balance between sampling error and the cost to attain a confident level of certainty.

Any sampling plan should require that the random sample is large enough to represent the entire lot of feed. The allowable GM threshold will affect the sampling regime, with lower threshold limits requiring larger sample sizes to achieve a set confidence level (Gilbert, 1999). Further, the robustness and reproducibility of the analytical method used to test for the presence of GM material will affect the necessary sampling strategy (Remund et al., 2001). The United States Department of Agriculture's Grain Inspection, Packers, and Stockyards Administration personnel refer to the USDA Grain Inspection Handbook for guidelines for sampling to test for the presence of biotechnology-derived grains and oilseeds (USDA, 2005). The effectiveness of applying sampling protocols that were not specifically designed for detection and quantification of contaminating GM materials, however, may be compromised if they do not account for non-uniform and non-random distributions. This is likely the way in which adventitious GM products will be present in a shipment. Paoletti et al. (2003) have described the effect of heterogeneity on estimating the GM content of a kernel lot and have found it to become increasingly unstable as the degree of heterogeneity increases.

The method used to select laboratory samples can also be a significant source of error in the detection of GM feed. Typically, a field sample taken from a large bulk source such as a truck is sub-sampled and then that sample is ground to a fine powder. Depending on the analytical test to be performed, a sub-sample from this powder or the entire powder is used for analysis as a test sample. For example, some lateral flow tests can accurately detect 1 GM maize kernel in 800 kernels and may use a 240 g sample for testing (EnviroLogix Inc., 2005). For more sensitive methods such as polymerase chain reaction (PCR), 100–200 mg of test sample may be enough material for analysis. In the latter assay, a further sample step is required (Lipp et al., 2005). Only a sample of the total extracted DNA is used in PCR. Typically, this amount is 100–200 ng of DNA. The initial sub-sample size and number of repeats should be determined with consideration of the precision of each method used. Hubner et al. (2001) described a laboratory sample scheme for the detection of GM maize and soybean by quantitative competitive PCR (QC-PCR) and real-time PCR (RT-PCR) methods. The precision, expressed as relative standard deviation, ranged from 5 to 20%. With respect to this, an overall sampling error of 20% was assumed to be acceptable and a laboratory sample containing a heterogeneous distribution of GM material needed to contain 10,000 particles, or seeds or kernels depending on the sample, when a threshold value of 10 g/kg was considered. The lab sample size then depends on the crop type and could vary

between 40 g for canola to 2850 g for maize. In the case of a homogenous distribution, only 3500 particles were considered to be necessary for analysis. Such calculations should be applied to detection assays for each GM crop so that confidence levels can be established.

2.3. Matrix effects

When DNA or proteins are isolated from a sample, other factors, including plant substrates, feed additives, or reagents used in extraction procedures, can be co-purified. The effects of these factors on analytical tests, if any, are called matrix effects (Stave, 1999). Typically, impurities in a plant or DNA matrix have adverse effects on sample analyses. Impurities are usually more critical to DNA analysis than to protein analysis, and can significantly affect the efficiency of detection. Plant polysaccharides and polyphenolics can inhibit the PCR or degrade DNA (Wilson, 1997; Demeke and Adams, 1992). Hexadecyltrimethylammonium bromide (CTAB) and isopropanol are examples of chemicals commonly used in the extraction of DNA that are known to inhibit PCR at concentrations greater than 0.05 mg/ml (w/v) and 0.01 ml (v/v), respectively (Peist et al., 2001).

Foods and feeds provide some of the most complex matrices for isolation and detection of protein or DNA analytes. The combination of multiple plant substrates and addition of other ingredients such as salts, along with heat processing, may yield unexpected matrix effects. It is important to test for these effects when standardizing procedures, as they can affect the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ are the concentrations of the least amount of analyte that can be reliably detected or quantified, respectively. Quantification of analyte should not be attempted below the LOQ (Hubner et al., 2001), which is typically 5–10 times the LOD (Holst-Jensen et al., 2003). For DNA-based detection methods, the LOD and LOQ are dependent on genome size (Van den Eede et al., 2002) and the number of transgene inserts per genome, whereas LOD and LOQ of proteins are affected by the level of expression (Stave, 2002). Both assays can also be dependent on the type of matrix present, extraction efficiency, and sensitivity of the test. To determine whether a matrix alters either the LOD or LOQ, a comparison can be made between an analyte extracted from a feed sample with a known content of GM plant and a standard obtained separately from the sample (Stave, 1999). Standards can be produced from *in vitro* methods, such as replication of plasmid DNA or the expression of protein in bacteria, or they may be derived from a single GM plant (total DNA or protein isolated from one plant). Alternatively, a known amount of GM standard can be introduced into isolated DNA or the protein matrix that came from a feed containing non-GM parental plant. Differences between detection or quantification of the recombinant analyte with and without the matrix can then be determined (Stave, 2002).

2.4. Reference materials for quantification

Validated reference materials are important for implementing detection systems and standardizing testing between laboratories. Certified reference materials (CRMs) for Roundup Ready® soybeans, Bt-176 maize, Bt-11 maize, and MON810 maize, encompassing varying percentages of GM content on a whole weight basis, are available from the Institute for Reference Materials and Measurements (IRMM, 2004). The CRMs are available as powder

forms and are diluted with identical non-GM crops, therefore representing a single matrix. Ideally, this makes the CRMs useful for testing products of a single plant type. However, even for single matrices, the powder CRMs may not be suitable reference material for GM crop quantification given that DNA and protein levels may vary on a weight percent basis due to factors such as variety and growing conditions, which could produce error when analyzing test samples (Holst-Jensen et al., 2003). Additionally, any difference in particle size between reference standards and test samples could add significantly to the overall error (Prokisch et al., 2001). This is because the concentration of analyte can vary in different particle fractions of biological materials. For example, DNA content is not homogenous throughout a maize kernel and particles from the embryo are likely to have higher DNA concentrations than those from the endosperm (Trifa and Zhang, 2004). In addition, the difference in particle size will likely affect the efficiency of extraction, with smaller particles providing greater surface area and better overall extraction. The ideal particle size for similar DNA distribution is between 10 and 100 μm (Prokisch et al., 2001).

Particle size is also critical to proper protein quantification. Diaz et al. (2002) reported that Cry9C protein expression occurred differently within various constituents of a maize kernel, with the greatest concentrations occurring in the endosperm and hull. In a collaborative study involving 40 international laboratories, an enzyme-linked immunosorbent assay (ELISA) for quantifying the concentration of MON810 maize in unknown samples was tested (Stave et al., 2000). The standards used for the ELISA consisted of maize flour prepared at a commercial plant. Two sets of unknown samples were tested by each lab. The first set included flour of varying concentrations (w/w) prepared at the same plant as the standards. The second set comprised CRMs produced by the IRMM, which consisted of ground whole maize. The concentrations of the flour unknowns ranged from 0.97 to 0.99 of the actual concentration. In contrast, the concentrations of the CRMs were overestimated by 14–24%. The authors attributed the overestimation to particle size of the standards (150 μm) compared to the CRMs (35 μm), which could have resulted in over-recovery of the Cry1Ab protein from the CRMs. This study highlights the importance of using similar extraction procedures for both standards and test samples analyzed by ELISA when using whole solid plant substrate as the standard.

Testing a sample containing a single type of feed product provides the simplest situation for the detection of GM ingredients and occurs for bulk, raw products. If a proper identity preservation system is in place, then the need for repeat verification of processed feeds is reduced (Brookes, 2002). However, regulators may test processed food and feed products to ensure that producers are complying with labeling laws (Stave, 2002). Should the need arise to test an end product such as a diet containing more than one plant component, it would be ideal to have a reference for that diet. This is because the matrix of the diet may affect the efficacy of a test differently than would a single GM product. For example, the concentration of tannins and other polyphenolic compounds, known inhibitors of PCR, may vary substantially among diets. The extent to which inhibitors will affect PCR depends on the effectiveness of the DNA purification procedure employed during sample analysis. One study compared six different extraction methods for the detection of DNA from transgenic maize (Holden et al., 2003). Two of the methods involved binding of DNA to silica gel, one used magnetic beads to bind DNA, another consisted of cell lysis and protein precipitation, while the last two utilized a selective DNA precipitation step, conducted either with or

without CTAB. DNA yields varied among the methods, as did purity of the DNA, which was determined by spectrophotometric and by PCR analyses. The authors found that employing DNA isolation methods involving binding to a solid matrix (silica or magnetic particles) or selective DNA precipitation with CTAB were most conducive to the successful PCR amplification of transgenes.

Alternatively, extracted DNA or proteins in standardized solutions may serve as useful reference materials for GM feed detection and quantification. The merit of such standards would have to be tested against each type of feed to account for differences in matrix effects among diets. Furthermore, processing feed materials might change the overall composition of the product and/or alter the DNA and protein content and structure. The usability of standardized solutions is also improved compared to commercial CRMs because standards with a GM content of any concentration can be formulated (Pardigol et al., 2003). Accurate quantification of test samples is limited at the 50 g/kg level with some CRMs because concentrations greater than 50 g/kg are not available. A drawback of such reference materials, however, would be that the extraction procedure and matrix could differ from the test samples. This could be partially overcome if the reference DNA or protein were concentrated and therefore could be introduced into matrices lacking GM analyte for design of standards exhibiting matrix effects similar to the test samples. Kuribara et al. (2002) synthesized two unique plasmid standards to quantify either GM maize or soybean by RT PCR. The maize plasmid contained sequences specific to five types of GM maize, the Cauliflower Mosaic Virus (CaMV) 35S promoter and nopaline synthase terminator for general screening of GM plants, and the endogenous maize starch synthase IIb gene. The soybean plasmid was similar except it was specific to only 1 GM variety and contained a region of the soy lectin endogenous gene. Although diluted in purified salmon DNA instead of plant matrix, the standards were effectively used for quantification of maize and soy samples in a validation study (Shindo et al., 2002). The use of plasmid DNA as standards however is limited by accurate quantification of the plasmid due to its small size (Lipp et al., 2005). In addition, plasmid DNA poses a contamination problem in the laboratory and could potentially lead to false positive results (Lipp et al., 2005).

2.5. Detection of GM plants by protein-based methods

Detection and quantification of novel proteins expressed in GM crops are accomplished by immunoassays. The two most applicable immunoassays concerning large scale GM detection are lateral flow strip tests and ELISA. Commercial varieties of both are available for the detection of numerous GM crops expressing proteins that confer insect resistance or herbicide tolerance. Although western blots are highly sensitive and have LODs of 2.5 g/kg for Roundup Ready® soybean seeds and 10 g/kg for toasted soybean meal (Rogan et al., 1999), the time and cost requirements of this type of assay limit its usefulness.

Lateral flow strips utilize a double antibody system to detect novel plant proteins in bulk and some processed feeds. The strips contain excess antibodies (Ab) coupled to a color reagent and are specific to a recombinant protein. Lateral flow strips are highly sensitive and some commercial varieties can detect as little as 1 GM maize kernel in 800 or 1.25 g GM/kg. Although they are qualitative in nature and provide only a positive or negative outcome, results can be obtained in less than 10 min and can be performed easily in the field. They can

be used for quality control to quickly ascertain which shipments of plant materials contain an engineered trait.

However, test strips do appear to be subject to user error. A study conducted to examine the ability of test strips to detect GM soybean in a grain handling facility reported substantial variation in detection, depending on the concentration of GM soybean in the sample (Fagan et al., 2001). When unknown samples contained 0–10 g GM/kg, the frequency of false positives was 0.067 whereas the frequency increased to 0.223 when the samples contained 0–100 g GM/kg. The higher rate of false positives for the 100 g/kg samples was likely due to cross-contamination. On the other hand, false negatives occurred at frequencies of 0.677 and 0.682 when the actual GM contents were 5 and 10 g/kg, respectively. However, at a content of 100 g GM soybean/kg, all samples were correctly identified as GM. The study highlights the importance of training field personnel proper techniques to avoid contamination, which are usually in place within laboratory settings.

In contrast to lateral flow strips, ELISA can provide quantitative data. An international study involving 38 laboratories showed the capability of ELISA to detect GM soybean expressing the recombinant protein CP4 EPSPS (Lipp et al., 2000). The experiment was designed to determine if Roundup Ready® soybean could be detected above or below a threshold set at 20 mg/g. The test samples contained GM soybean powder at 0 to 20 mg/g (in conventional soyabean powder). The ELISA had a LOD of approximately 3.5 mg/g. When the sample concentration was less than 20 mg GM plant/g, the ELISA identified the sample as being less than 20 mg/g with a 99% confidence level. In addition, quantitative use of the assay resulted in good repeatability ($R.S.D._r = 7\%$) and reproducibility ($R.S.D._R = 10\%$). Both $R.S.D._r$ and $R.S.D._R$ are measurements of precision within the same lab and between labs, respectively. Assays for quantification of molecules within biological matrices are generally given an acceptable limit of 15% R.S.D. at a particular concentration level unless the concentration is at the lower limit of quantification, in which case it should not exceed 20% R.S.D. (U.S. Department of Health and Human Services, 2001).

Although these immunochemical tests can provide useful data on identity preservation and screening for the presence of GM crops in test samples, their usefulness for detecting specific GM events is limited. For example, Bt176, Bt11 and MON810 events all result expression of the Cry1Ab protein, to varying degrees. Thus, protein (Cry1Ab) detection will not indicate the concentrations of these individual GM crops within a mixture. In addition, the rates of expression of novel proteins will vary among tissues of a plant and with the maturity of the plant (Stave, 2002). In certain applications, therefore, use of protein standards diluted on a w/w basis will not yield useful information.

2.5.1. Feed processing

Immunological assays can be used for quantitative and qualitative analyses of bulk raw materials. They may become limited however, when feeds or foods are processed or testing is required on an unknown mixture of ingredients containing a complex matrix, which is typical of processed foods or feeds combined as a total mixed diet. The accuracy and precision of immunological assays can be diminished by the potential interaction of specific Ab with matrix proteins, saponins, or phenolic compounds (Anklam et al., 2002). Additionally, proteins denature at the high temperatures employed during feed processing, which can

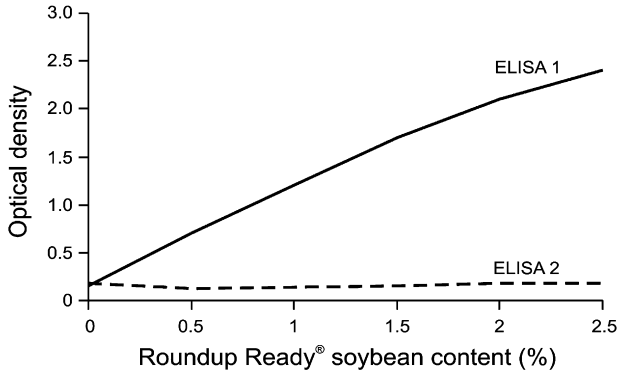

Fig. 1. Reactivity of two different ELISAs to the CP4 EPSPS protein in Roundup Ready® toasted meal. Antibodies in ELISA 1 were designed to be specific to the denatured form of CP4 EPSPS found in toasted soymeal. Antibodies in ELISA 2 were specific to normal CP4 EPSPS in Roundup Ready® soybeans. Adapted from Stave (2002). Printed with permission from J. AOAC Int.

change the tertiary structure and thus the epitopes recognized by Ab. Diaz et al. (2002) reported that 0.983–0.991 of the Cry9C protein in Starlink™ maize is denatured or degraded during the processing of maize. In contrast, Rogan et al. (1999) showed that processing of Roundup Ready® soybeans to toasted meal did not substantially affect the sensitivity of detection of either ELISA or western blot procedures, as the LOD increased from only 2.5 to 14 g/kg and 2.5 to 10 g/kg, respectively. The differences between the two studies may have been due to the Ab used, type of protein, or the nature of processing employed. Heat denaturation however, can be overcome by using Ab specific to a heat-resistant epitope or designing Ab to the denatured form of the protein (Stave, 2002). Fig. 1 shows how the design of Ab can affect recombinant protein detection by an ELISA after feed processing. Currently, Strategic Diagnostics Inc. produces lateral flow strips that have a LOD of 9 g/kg to test for CP4 EPSPS protein in toasted soy meal samples in 10 min. The company also manufactures quantitative ELISA plates that will detect 1 g CP4 EPSPS protein/kg in toasted meals, flour, soy milk, and tofu.

While heat denaturation of proteins may be overcome by designing Ab specific to the denatured protein, some feeds may be more limited to detection by immunological assays. Folmer et al. (2002) reported the effect of ensiling on the amount of Cry1Ab protein in earlier-maturing and later-maturing varieties of insect-resistant maize, both derived from event Bt11. The concentrations of Cry1Ab protein in fresh pre-ensiled plant material were 4923.5 and 8508.8 ng Cry1Ab/g dry matter for the earlier-maturing and later-maturing varieties, respectively. The amount of Cry1Ab decreased to unquantifiable trace levels after 9 days of ensiling for the earlier-maturing hybrid and 4 days for the later-maturing hybrid. Whether an immunological test can be tailored specifically to silage has not been reported. Jennings et al. (2003a) used a competitive assay to test for the CP4 EPSPS protein in pig tissues and showed that their assay was still highly sensitive even after partial digestion of the novel protein in simulated gastric fluid. Perhaps a competitive assay would increase the duration of recombinant protein detection in silage as well.

2.6. Detection of GM plants by DNA-based methods

In contrast to protein detection methods, DNA analysis can provide event-specific results for plants expressing the same recombinant protein. In addition, DNA-based tests will allow detection and quantification of GM plants that do not express any novel proteins, modified simply to repress gene expression. Gene detection is dependent on the ability to detect and quantify unique sequences of transgenic and endogenous plant DNA. Often, the starting concentration of a gene isolated in DNA is low, thus amplification of the target sequence is required. Therefore, DNA-based methods for detecting GM crops are usually PCR-related. Four categories of PCR to test for GM plants have been described and each depends on the sequence of DNA amplified (Holst-Jensen et al., 2003). The categories range from general screening of DNA sequences common to multiple GM plants, to event-specific detection of the junction between plant genomic and transgenic DNA. The latter sequence is unique to each GM plant. Critical to PCR is the design of highly precise primers so that non-specific DNA amplification does not result in false positives.

Conventional PCR can be used as a qualitative method to determine whether or not transgenic plant DNA is present in a sample and can identify the transgenic DNA associated with a specific transformation event. PCR is capable of detecting GM feed below the thresholds set by the European Union. Jankiewicz et al. (1999) were able to detect GM soybeans and maize in their respective 1 mg/g CRMs, which was the lowest concentration of CRM available for each GM plant. By serial dilution of extracted DNA however, the theoretical LOD was 0.05 mg/g for both GM plants in the presence of 1000 ng non-GM background plant DNA. Instead of diluting DNA, Van Duijn et al. (2002) diluted CRMs for GM soybeans to obtain powder mixes of 0, 0.1, 1, 5, 10, and 20 mg/g. The LOD was determined to be 1 mg/g by PCR. It is probable that serial dilutions of DNA differ for powder samples due to the variation in the extraction efficiency of DNA.

Validation studies have been completed to test PCR for detection of GM plants in powder CRMs and processed foods. A collaborative study involving 29 laboratories tested the detection of GM soybean and maize by qualitative PCR (Lipp et al., 1999). Samples containing 20 mg transgenic soybean or maize/g were identified as positive by all laboratories. Two PCR assays were used to detect GM soybean, one that targeted the NOS terminator and one that targeted the CaMV 35S promoter. Both were highly sensitive, providing correct identification of 1 mg CRM transgenic soybean/g 0.949 and 0.928 of the time, respectively. The maize assay was less sensitive, having only correctly identified a 1 mg CRM/g sample as positive 0.841 of the time. However, all assays had over 0.95 correct identification for the 5 mg/g CRMs. The lower sensitivity for maize was attributed to its larger genome size compared to soybean. Three false positives were found out of the 310 samples that were analyzed. Another validation study involving 23 laboratories investigated the detection of GM maize and soybean in processed foods (Lipp et al., 2001). The foods tested (polenta, acidified soybeans, infant formula, and biscuits) contained 0, 0.2, or 1.00 of GM product that represented either the entire food or was amongst other ingredients. All of the foods were processed at temperatures reaching at least 100 °C. Lipp et al. (2001) targeted the same genes as were tested in the study by Lipp et al. (1999), however primers amplifying shorter sequences of transgenic DNA (123 bp for the CaMV 35S promoter and 118 bp for the NOS terminator) were used to account for the likely DNA fragmentation during processing. In

total, the average frequency of false positives for foods containing 0 GM content was 0.03. Detection of GM material was correctly reported with an average frequency of 0.98. The study therefore showed that PCR assays are capable of detecting GM constituents in foods processed under extreme conditions. The same is likely true for processed feeds as well, which may also have sustained physical damage to DNA. The authors noted however, that extreme caution is necessary to avoid false positives resulting from contamination.

Although PCR validation has not yet been performed on animal feed samples, numerous studies investigating the fate of transgenic DNA throughout the digestive tract of livestock have shown the usefulness of PCR for detection of GM material in complex samples. Detection of GM feed in diets containing multiple plant species or after feed processing has been reported as described below although in most studies, LOD was not reported. Sharma et al. (2004), were able to detect 12.5 pg of transgenic canola seed DNA diluted in 100 ng of DNA from a complex matrix containing a processed diet after incubation in ruminal fluid. Similarly, Jennings et al. (2003a,b) showed the sensitivity of PCR analyses for transgenic DNA from herbicide-tolerant soybean in 10 μg of pig genomic DNA. By PCR, the LOD was approximately 25 pg, however this was enhanced to 2.5 pg by combining PCR and Southern blot analysis.

Because screening for all GM plants is laborious and costly and will likely become more so as new lines are approved, some multiplex PCR assays have been developed. Multiplex PCR allows for the simultaneous amplification of more than one target in a PCR reaction. For qualitative PCR, the products are then differentiated by size on a gel.

Hernandez et al. (2005), Permingeat et al. (2002) and Matsuoka et al. (2001) have designed multiplex PCR assays to screen for separate GM crop events (GTS 40-3-2, Bt11, MON810, T25, and GA21). The reactions were sensitive to at least 5 pg transgenic DNA/ng non-GM DNA. Furthermore, a seven-target multiplex PCR has been designed for four GM maize cultivars and one GM soybean cultivar, plus endogenous control genes for each plant species (Germini et al., 2004). The LOD of the assay was 2.5 pg GM/ng and the PCR proved effective for feeds and commercially purchased foods that had been processed.

Real-time PCR is a highly sensitive, preferred method for quantitative DNA analysis. Unlike conventional PCR, which measures products at the end of the reaction, RT-PCR quantifies DNA by fluorescent emissions released throughout the reaction during each amplification cycle. The most useful RT-PCR assays are those that use fluorogenic molecules specific for the target amplicon and will only emit a fluorescent signal as a result of directly or indirectly binding to the target. Highly specific RT-PCR does not require post-PCR processing, as the results are obtained throughout the reaction.

RT-PCR quantification is generally based on a standard curve prepared using known amounts of a target. There are two approaches to quantification using a standard curve and both compare the amount of transgenic DNA to reference DNA (Lipp et al., 2005). The first utilizes known amounts of target DNA (wt) or by inference, known amounts of target gene (copy number). The DNA amount in the unreplicated, haploid nuclear genome of an organism is known as its 1*C* value. An extensive database on plant *C*-values has been summarized by Bennett and Leitch (2004). DNA amounts for *C*-values can be expressed by weight or nucleotide base pair size (1 pg = 980 Mbp). If the genome of the plant is known and the number of copies of target gene per genome is known, then the number of gene copies in a PCR reaction can be calculated. For example, the average 1*C* nuclear value for

maize (*Zea mays*) is 2.6 pg (Bennett and Leitch, 2004). Assuming one transgenic construct per genome of maize, a standard curve encompassing 10, 100, 1000 and 10,000 copies of that transgene would need 26, 260, 2600, and 26,000 pg of maize DNA. In essence, the standards consist of diluted DNA. Measuring the percentage of GM plant in a sample by this method requires a test for the transgene as well as for a gene specific to the crop species. By quantitating the transgene and endogenous gene in equal amounts of DNA, and taking into consideration the copy number of each gene per genome, the percentage of GM DNA present can be calculated from the ratio of these figures. For example, if 100 copies of a transgene are detected and 200 copies of an endogenous gene are present in the same sample, then the proportion of GM plant present is 0.50, so long as both genes are present at 1 copy per genome.

The second type of standard curve enables quantification based upon differences in C_T (threshold cycle). By this method, the standards could be equal amounts of DNA extracted from diluted powders (w/w) containing different concentrations of GM plant material such as CRMs. Each PCR reaction would contain equal amounts of DNA but the curve would be generated from the differences in C_T values of the transgene and an endogenous gene resulting from each standard. Thus, the type of standard curve utilized would depend on whether quantification is desired in terms of gene copy equivalents or on the basis of weight of plant material.

Several studies have shown the capability of RT-PCR to quantify GM material in single seed samples below the EU threshold. A lower LOQ of 1 mg/g has been reported for GM rapeseed (Zeitler et al., 2002) and soybean (Huang and Pan, 2005) and assays with upper LOQ of 1000 mg/g have been designed for maize (Huang and Pan, 2005) and soybean (Huang and Pan, 2004). One interlaboratory study involving 22 laboratories was conducted to evaluate RT-PCR for determining the percentage of Roundup Ready® soy in soya flour by using a standard curve based on differences of C_T values (Hird et al., 2003). The PCR assay used primers specific for the transgene and an endogenous gene coding for lectin in soybean. The percentage of GM soya in the samples was calculated by using a matrix-matched standard curve derived from CRMs. The repeatability ranged from 9.3 to 19.3% $R.S.D._r$ and the reproducibility resulted in 20.3–33.7% $R.S.D._R$. Another study involving 13 laboratories tested reference plasmids designed for quantifying gene copy numbers from GM maize and soybean (Shindo et al., 2002). The assay generated results similar to the CRMs and had repeatability and reproducibility of quantification less than 18.9 and 18.8%, respectively, when the actual GM content was 10 mg/g. Although validation has not yet been reported for complex feed diets, RT-PCR has been applied to silage (Einspanier et al., 2004), commercial feed (Novelli et al., 2003) and a pelleted mixed diet (Alexander et al., 2004).

As with detection of proteins, feed processing could affect DNA assays. However, DNA is more resilient to processing than proteins. Matrix effects may be more critical to proper DNA analyses. Novelli et al. (2003) analyzed 88 samples of mixed diets for cattle developed and provided by several agro-industrial companies by screening for the CaMV 35S promoter. In several cases, one method of DNA isolation resulted in an inability to detect the targeted region within the promoter, whereas isolation of DNA by a different method returned positive results, illustrating the requirement to remove PCR inhibitors to ensure accuracy of the assay. In the same study, the proportion of GM content in some of the feed samples was quantified but it was not reported if the type of extraction affected quantifi-

cation. Peano et al. (2004) compared four methods of DNA extraction and found that the type of extraction significantly affected the standard curves of the PCR assay. Therefore, the extraction method should be uniform for the standards and any test samples.

2.6.1. Feed processing

Gawienowski et al. (1999) described the fate of endogenous maize DNA during steeping, wet-milling, and processing. Both nuclear and chloroplast *rubisco* genes were analyzed by PCR. The genes were amplifiable in starch, germ, coarse fiber, and wet gluten samples after wet-milling and steeping. However, *rubisco* genes were not detectable after drying the gluten at 135 °C for 2 h. After industrial scale processing, the DNA sequences were amplifiable from maize meal. Similarly, the genes were detected in wet gluten and gluten fractions used for the production of feed pellets. However, the heating process involved in feed pellet production appeared to degrade intact DNA to undetectable levels. Similarly, Chiter et al. (2000) reported that the treatment of oilseed rape meal (canola) resulted in complete degradation of DNA and that heating maize grains to 95 °C for 5 min resulted in an inability to amplify a 577 bp gene sequence by PCR. In contrast to these studies, high molecular weight (23 kbp) DNA has been reported to remain after the solvent-extraction of canola seed, both in the meal and after pelleting of canola meal into complete diets (Alexander et al., 2002). Throughout canola meal and diet processing, temperatures reached up to 100 °C. A 540 bp fragment of endogenous DNA was detectable in both parental and Roundup Ready® canola leaves, seeds, meals, and diets. The entire 1363 bp *cp4 epsps* transgene was also amplifiable from all transgenic substrates but not the parental control samples (Fig. 2). The discrepancies in the above studies may have resulted from differences in the duration of heat processing of the specific gene fragment used for detection. While

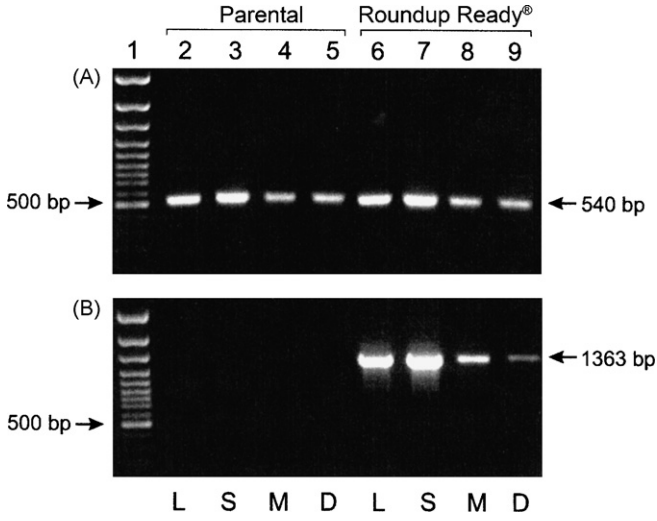

Fig. 2. PCR analysis of DNA fragments from parental and glyphosate-tolerant (Roundup Ready®) canola leaves (L), seeds (S), meals (M), and diets (D) derived from the meals. (A) Presence of 540 bp canola-specific *rubisco* fragment. (B) Analysis of 1363 bp *cp4 epsps* transgene. From Alexander et al. (2002).

qualitative detection of transgenic DNA after feed processing may be possible, the ability to quantify GM content may be diminished due to DNA fragmentation. Yoshimura et al. (2005) tested the effect of heat treatment on the quantification of transgenic and endogenous DNA in ground GM maize and soybean seeds. The samples were autoclaved in water for 0, 5, 15, 30 and 60 min at 110 °C. Several primer and probe sets were used to quantify the genes. When the size of the amplicon for the endogenous gene and transgene differed, the ratio of the prevalence of these genes changed substantially over time. However, the ratio of genes improved when both amplicons were of similar size. This suggests that the extent of degradation can differ for transgenic and endogenous DNA and highlights the importance of amplicon size in RT-PCR.

The size of a target DNA sequence is also important to other feed processes that result in DNA degradation, such as ensiling (Hupfer et al., 1999). During ensiling of Bt maize, a 1914 bp fragment of the transgene could only be detected up to 5 days. However, a 211 bp fragment of the transgene was amplifiable for up to 100 days of ensiling, as was a 226 bp fragment of an endogenous control gene. Furthermore, Aulrich et al. (2004) were able to detect a 194 bp fragment of the *Pat* transgene in whole plant silage and maize cob mixed silage over the entire duration (200 days) of ensiling. Amplification of smaller gene fragments therefore seems more suitable for detection of GM crops in silage. Einspanier et al. (2004) reported that the concentration of plant genes decreased to less than 0.03 of the original concentration after ensiling transgenic maize. To date, quantification of processed GM feed has not been reported. Both the complex matrices and feed processing can affect reliable quantification of GM feed. Therefore, validation studies aimed at quantifying GM content under such conditions are required.

3. GM feed and livestock

3.1. Rationale for animal studies

Although regulations with regard to GM plants have been developed primarily from the perspective of human consumption of GM food, it is generally assumed that these same criteria are suitable for a risk assessment of the consumption of GM feed by livestock. The European Commission has a combined safety approach that requires an assessment of risks for humans, animals, and the environment prior to approval of importation or cultivation of a novel crop (EC, 2001). In contrast, Canada has legislation relating specifically to the safety assessment of novel GM feeds. Under this legislation, all livestock feeds derived from plants with novel traits are subject to mandatory review for safety and efficacy by the Canadian Food Inspection Agency under the Feeds Act and Regulations (CFIA, 1995).

An integral part of the safety evaluation of GM plants is to test for substantial equivalence. The protocol for establishing substantial equivalence does not complete a nutritional safety assessment of a GM plant. Rather, it provides a starting point for the overall assessment (FAO/WHO, 2000). The aim of such a test is to determine whether a transgenic plant is substantially equivalent to its conventional counterpart at a chemical and nutritional level. While the parameters to be measured have not been formally defined, minimal analyses performed should determine whether the major nutritional components (*i.e.*, lipids, carbo-

hydrates, proteins, vitamins, minerals, trace elements) and known antinutrients and toxins of transgenic plants are equivalent to those in conventional varieties that have a history of safe use. For livestock nutrition, important measurements include crude protein, fat (ether extract), fiber, starch, amino acids, fatty acids, ash and sugar (Aumaitre et al., 2002). Some of these factors not only affect animal health and performance, but can also alter the composition and quality of animal products provided to the consumer. For example, certain fats present in feeds can affect the composition of fat in animal tissues (Aumaitre et al., 2002). Guidelines for second generation GM crops that are not substantially equivalent have been proposed (Flachowsky and Bohme, 2005).

Animal experiments are important and provide valuable information regarding the safety of a GM plant for both livestock and human consumption. Adverse effects of a plant fed to livestock should be a warning for human use. In addition, economic concerns regarding animal performance are also addressed. To date, no studies have demonstrated adverse effects in any of these areas when the currently registered GM plants or products derived from them have been used as feeds (Flachowsky et al., 2005). With regard to the GM traits presently available, it appears that the protocols for establishing substantial equivalence have also confirmed in nutritional equivalence. Nonetheless, a significant amount of research regarding the fate of recombinant molecules within GM crops after consumption by livestock has been conducted. Information on most of the plants with novel traits employed in the animal studies listed below are described by AGBIOS, 2005.

4. The fate of transgenic proteins

4.1. Rationale for investigating the fate of transgenic proteins

Guidelines have been established by several organizations regarding assessment of the allergenic risk of each novel protein expressed in a GM plant, prior to market approval (FAO/WHO, 2000; Martens, 2000; Konig et al., 2004). These typically include comparison of amino acid sequence homology of the novel protein to known allergens and digestion of the protein in simulated gastric environments. While allergic reactions are primarily a concern for human consumption of GM foods, certain proteins in soybean have been shown to elicit allergenic reactions in calves and piglets (Van Dijk et al., 1988). It is common practice to apply heat to some feedstuffs to inhibit the actions of anti-nutritional proteins. Such is the rationale for toasting soybean, which contains trypsin inhibitors and haemagglutinins. The fate of novel proteins in GM feed consumed by livestock has also generated interest arising from consumers' questions regarding the possible appearance of novel proteins in animal products entering the market for human consumption and the possible effects of such events on commercial trade.

4.2. Protein intake by livestock

The amount of transgenic protein ingested by livestock depends on the concentration of the protein in the feed and the amount of feed intake. Transgenic protein concentration varies with the transgenic event and the type of plant tissue in which it is expressed (Stave,

2002). For example, the concentration of Cry1Ab protein in the grain of event MON810 insect-resistant maize has been reported to be 0.31 μg/g of fresh weight tissue (fwt), whereas the same protein in event Bt11 maize grain was present at 4.76 μg/g (Stave, 2002). In event MON810 maize, Cry1Ab concentration in leaf tissue was 9.35 μg/g fwt, more than thirty times greater than in the grain. Environmental conditions under which plants are grown can also lead to varying concentrations of transgenic protein. One study showed that the average Cry1Ab content of MON810 leaves in 1995 was 8.6 μg/g fwt, compared to 12.15 μg/g fwt the following year (Monsanto, 2004). In addition, post-harvest feed processing can alter (likely decrease) transgenic protein concentrations, as discussed above. Anything affecting the concentration of transgenic protein in feedstuffs will also alter the total transgenic protein intake by livestock. Similarly, feed intake, which varies with animal and diet, will influence protein intake. Assuming intake of 10 kg of MON810 grain per day, total daily Cry1Ab intake by a dairy cow will be approximately 3.1 mg (10,000 g × 0.31 μg/g fwt = 3100 μg or 3.1 mg). Feed and protein digestion are also species-dependent. Thus, the presence of transgenic protein throughout animal digestive tracts and potentially in livestock products depends on multiple factors.

4.3. Ruminants

Chowdhury et al. (2003a) used an ELISA to quantify Cry1Ab protein throughout the digestive tract of calves fed insect-resistant maize kernels (event Bt11). The transgenic protein was detected in ruminal solids and aqueous supernatant, digesta from the abomasum, jejunum, colon and rectum, and in feces. The Cry1Ab concentration varied throughout different segments of the gastrointestinal tract (GIT) and even increased as a percentage of total protein upon passage from the abomasum and jejunum to the large intestine. The concentration of Cry1Ab in feces was similar to that in ruminal fluid and therefore the authors also tested the persistence of Cry1Ab in feces under ambient conditions in soil. At 14 days, the protein was no longer detectable. Whether or not recombinant proteins found in the excreta of livestock pose any ecological risk has not been tested. In the case of Cry proteins, marked effects are unlikely, as these have been used for many years in agriculture as pesticides (McClintock et al., 1995). Evidence of absorption of the 65 kDa Cry1Ab protein was not obtained from assays of tissue extracts including liver, spleen, kidney, mesenteric lymph node, and muscle (Chowdhury et al., 2003a). Similar findings have been reported in cattle consuming silage from a different type of insect-resistant maize (event Bt176; Einspanier et al., 2004). Cry1Ab protein was not detectable by ELISA in epithelial tissue from the abomasum, jejunum, and colon. However, Cry1Ab was quantifiable in contents from the rumen, abomasum, jejunum, colon, and feces. As observed by Chowdhury et al. (2003a), the concentration of transgenic protein in total protein isolated did not decrease during passage through the GIT. This was not likely attributable to the resistance of Cry1Ab to digestion, as all of the currently marketed Cry proteins have been shown to breakdown in gastric fluid in 0–7 min (Mendelsohn et al., 2003). Lutz et al. (2005) have recently determined why the Cry1Ab protein may appear to resist degradation during passage though the bovine GIT. They analyzed the same samples isolated from the study by Einspanier et al. (2004) in addition to new GIT samples from cows fed Bt176 maize. When the samples were analyzed with an ELISA specific to the Cry1Ab protein, the concentration appeared to increase

throughout the GIT. However, they attributed this to polyclonal Ab in the ELISA reacting to degraded fragments of Cry1Ab. When the samples were applied to an immunoblot assay using a monoclonal Ab, degradation of the Cry1Ab in the samples was apparent.

4.4. Pigs

The digestibility of the Cry1Ab protein throughout the digestive tracts of pigs fed diets containing 600 g Bt11 maize/kg has been determined (Chowdhury et al., 2003b). By ELISA, the amount of Cry1Ab protein in the diet was 600 ng/g of total protein and 300 ± 140 ng/g in rectal content. The protein was detectable in stomach, duodenal, ileal, cecal, and rectal contents of the animals and coefficient of digestibility of Cry1Ab was estimated to be 0.92. The half life of intact CP4 EPSPS in simulated gastric fluid has been calculated to be less than 15 s and less than 10 min in simulated intestinal fluid (Harrison et al., 1996). In another study (Jennings et al., 2003a), CP4 EPSPS protein could not be detected in the *longissimus* muscle of pigs fed herbicide-tolerant soybean meal (event 40-3-2). Those authors used a competitive ELISA that was capable of detecting partially digested CP4 EPSPS in a manner similar to that for intact CP4 EPSPS. The ELISA, which had a LOD of 94 ng of transgenic protein per gram of pork muscle, enabled the detection of CP4 EPSPS fragments, the most probable form in which the protein would be absorbed, but CP4 EPSPS was not detected in any of the muscle samples.

4.5. Poultry

A competitive ELISA similar to that used by Jennings et al. (2003a) was used to investigate the presence of Cry1Ab protein in the breast muscle of chickens fed diets containing insect-resistant maize (event MON810; Jennings et al., 2003b). The assay, which had a LOD of 60 ng Cry1Ab protein per gram of chicken muscle, again proved useful in detecting partially digested transgenic protein after exposure to a simulated gastric environment. Fragments of Cry1Ab were not detected in any of the tissues assessed. Furthermore, Yonemochi et al. (2002) investigated the transgenic Cry9C protein in tissues from chickens fed maize (event CBH 351) and were not able to detect the novel protein in blood, liver, or muscle samples. Ash et al. (2000) also did not detect transgenic protein in chicken tissue. Whole egg, egg white, liver, and feces all tested negative for the presence of CP4 EPSPS protein in laying hens fed a diet containing Roundup Ready® soybean meal. CP4 EPSPS was detectable in the meal and diet, suggesting that some of the protein remained even after processing.

5. The fate of transgenic DNA

5.1. Rationale for investigating the fate of transgenes

In contrast to the safety assessment of proteins, which is based mainly on tests measuring direct effects, concerns regarding recombinant DNA are based on indirect consequences resulting from possible transformation events. The fate of transgenes has been studied to a greater extent than that of proteins because of more issues concerning human health. It has

been suggested that the CaMV promoter, which is a regulatory sequence common to most registered GM plants, could cause cancer through over-expression of oncogenes, should the promoter be integrated into human cells through recombinant events after absorption (Ho et al., 1999). However, CaMV is ubiquitous and its promoter has been detected in food samples that do not contain transgenic DNA from GM plants (Wolf et al., 2000). The FAO and WHO have both stated that there is no direct health risk to consumers ingesting transgenic DNA because the DNA from all organisms is structurally similar (WHO, 1991). As with transgenic proteins, however, the presence of transgenes in animal products could also affect commodity sales. Another concern regarding transgenic plant DNA that has been raised is the possible transfer of antibiotic-resistance markers (ARMs) to bacteria. However, the ARMs used in currently registered GM plants are unlikely to result in the development of resistance to the therapeutic antibiotics presently used in animal and human health (FAO/WHO, 2000). The antibiotics used as markers are either rarely used in human medicine or are those to which widespread resistance is already prevalent in nature. A recent review has suggested that ARMs in GM plants do not pose a significant threat to human health (Gay and Gillespie, 2005). The FAO/WHO (2000) have proposed that for the stable transfer of plant DNA into a microbial or mammalian cell to occur, all of the following events would be necessary:

- the relevant gene(s) in the plant would have to be released, probably as linear fragments;
- the gene(s) would have to survive nucleases in the plant and in the gastrointestinal tract;
- the gene(s) would have to compete for uptake with dietary DNA;
- the recipient bacteria or mammalian cells would have to be competent for transformation and the gene(s) would have to survive their restriction enzymes;
- the gene(s) would have to be inserted into the host DNA by rare repair or recombination events.

It is important to note that the gene(s) would have to survive any feed processing events prior to intake by livestock as well. Moreover, aside from competing with other plant DNA, the gene(s) would be heavily diluted in microbial DNA and would have to compete with that DNA for uptake into a cell, especially in animals that harbour substantial digestive microbial populations upstream of gastric digestion in their GIT, such as ruminants and poultry.

Prior to the marketing of GM plants, there was little interest in the fate of plant DNA after consumption because the dietary requirement of nucleotides is usually met by the amount of DNA found in food (Beever and Kemp, 2000). It has been documented that DNA is reduced to smaller fragments, nucleotides, and nucleosides as a result of mastication, acid hydrolysis, and enzymatic cleavage during digestion (Armstrong and Hutton, 1974; McAllan, 1982; Beever and Kemp, 2000). However, knowledge about the fate of ingested feed DNA fragments was previously limited. In recent years, studies have added significant insight into the fate of plant DNA fragments after consumption.

5.2. Transgene intake by livestock

As for proteins, the amount of transgenic DNA ingested depends on the concentration of transgene in feed as well as feed intake. The quantity of DNA in most crops is less

than 0.2 g/kg DM (Beever and Kemp, 2000). As a percentage of total DNA, the transgene concentration is unlikely to change for an individual event because nuclear gene insertions are generally static. However, as a percentage of DM intake, the concentration of transgenic DNA can vary in response to environmental conditions that change the overall biomass of the plant, such as lignification with aging. Different parts of a plant will have variable transgenic DNA content as well, which is dependent on the quantity of cells per gram of DM. In addition, DM transgene content can differ significantly between events of the same crop variety because the genome size of crops is not constant and can show variation of up to 25% (Van den Eede et al., 2002). Beever and Phipps (2001) estimated that a dairy cow consuming 24 kg DM/day of a diet containing 400 g transgenic maize silage and 200 maize grain/kg, would have an intake of 57 g/day of total plant DNA. Of that, 54 μg would be recombinant DNA and account for only 0.00000094 of the total DNA intake. The actual total transgenic DNA intake may actually be lower, considering that ensiling GM plants quickly leads to degradation of large plant DNA fragments (Hupfer et al., 1999). Other processes too, such as heat treatment, will also lower the intake of intact transgenes.

5.3. Ruminants

The first studies investigating the fate of plant DNA in ruminants took place more than three decades ago and showed that most plant DNA, whether fed as a free form or as whole plant, is quickly degraded to oligonucleotides, nucleosides and bases throughout the digestive tract (Smith and McAllan, 1971; McAllan and Smith, 1973; McAllan, 1980; Razzaque and Topps, 1981). These studies focused on total nucleic acids and did not attempt to describe differential plant gene digestion in part because this was not of concern at the time and also because the molecular techniques to do so were yet to be developed. Subsequent to the introduction of GM plants, numerous reports have attempted to describe the persistence of transgenes.

Deoxyribonuclease activity has been shown to be present in bovine ruminal fluid (Flint and Thomson, 1990; Duggan et al., 2000; Ruiz et al., 2000) and ovine intestinal fluid (Alexander et al., 2004). This likely explains why detection of most plant DNA genes, at least those present at low copy numbers, have been in association with feed residue. Phipps et al. (2003) analyzed gene stability throughout the digestive tract of dairy cows fed diets containing (per kg) 185 g insect-resistant maize (event MON810) and 130 g herbicide-tolerant soybean meal (event GT 40-3-2). In the liquid phases of both ruminal and duodenal fluids, only a 167 bp sequence of the high copy chloroplast *rubisco* gene was detected. In contrast, none of the low copy amplicons from the endogenous soybean *lectin* (240 bp) and recombinant (171 bp) genes or from the endogenous maize *HMP* (209) and recombinant (203 bp) genes could be detected. All of the fragments were amplifiable from the solid phases of each digesta fraction. In the feces, only the *rubisco* gene was detected. Similar results were reported for the 1363 bp *cp4 epsps* transgene found in herbicide-tolerant canola (event Gt73) when canola substrates were incubated in ruminal batch cultures (Alexander et al., 2002). The same was also true for smaller fragments of the transgenic construct in Gt73 canola substrates, ranging in size between 300 and 527 bp, when incubated in ruminal batch cultures (Sharma et al., 2004). A 62 bp sequence of the transgenic construct however, has been detected in the aqueous phase of intestinal fluid *in vitro*, but the copy number of

this amplicon peaked at only 1600 copies when digestion was at its greatest (Alexander et al., 2004). The small amplicon size likely contributed to these findings, as the entire 1363 bp transgene was not detected in the liquid phase.

Einspanier et al. (2004) used real time PCR to quantify transgenic and endogenous genes throughout the digestive tract of cattle fed diets containing 885 g Bt176 maize silage/kg. After ensiling, the quantity of each gene decreased to less than 0.03 of the starting quantity. Surprisingly, the amounts of both transgene and endogenous gene seemed to increase after passage from the rumen to the abomasum, before decreasing dramatically to unquantifiable levels in the jejunum and colon. It should be noted that the quantity of each gene was expressed per 90 ng of total DNA. Therefore, it is likely that the plant genes were diluted with microbial DNA to a greater extent in the rumen than in the abomasum. Both the transgene and endogenous gene followed similar trends throughout the GIT.

The likely site for absorption of plant DNA fragments, should it occur, would be the intestine, and more specifically, the Peyer's patches of the distal ileum or proximal large intestine (Schubbert et al., 1997). Because of the highly unstable nature of DNA in the ruminant digestive tract, it is probable that DNA released in the rumen, at least for low copy genes, does not persist to the proximal small intestine. Therefore, passage of plant residues into the ileum prior to their digestion may be necessary for plant genes to have a chance of crossing the intestinal barrier. There is evidence that digestion does occur in the ileum (Erfle et al., 1982; Alexander et al., 2004) and that plant DNA is released into the aqueous intestinal phase. The first study to probe for plant DNA in ruminant products showed that transgenic DNA was not detectable in the muscle, liver, spleen, kidney, or blood lymphocytes of cattle given *ad libitum* access to Bt 176 silage (Einspanier et al., 2001). Given the reduction in transgene concentration during ensiling, these results are not surprising (Einspanier et al., 2004). A 199 bp chloroplast sequence of DNA, however, could be detected in the blood lymphocytes. Similarly, a *rubisco* gene fragment was detected in the blood of cattle fed GM maize and soybean meal, but transgenic sequences were never detected (Phipps et al., 2003). The same occurred for calves fed rations containing Bt11 insect-resistant maize at 433 g/kg (Chowdhury et al., 2004). A 231 bp fragment of the *rubisco* gene was detected in the liver, spleen, kidney, mesenteric lymph nodes, and longissimus muscle samples. However, the c*ry1Ab* transgene tested negative in all of the tissue samples. Nemeth et al. (2004) detected a 173 bp sequence of the *rubisco* gene in the beef brisket muscle of cattle fed a diet containing (per kg) 750 g dry rolled maize and 150 g maize silage (event MON810) at a frequency of for 0.05, whereas transgenic DNA was not detected. The same authors also tested for plant DNA in milk from dairy cattle fed a diet containing (per kg) 200 g maize plus 600 g maize silage of the same MON810 event. A 173 bp sequence of the *rubisco* gene was amplifiable in 0.86 of the samples, and indeterminate in the other 0.14. A larger 500 bp sequence of the same gene could be detected in 0.79 of the samples, with the remaining 0.21 indeterminate.

Castillo et al. (2004) fed insect-resistant cotton seed (transformed with *cry1Ac* or with c*ry1Ac* plus *cry2Ab*) or two varieties lacking the c*ry1Ac* transgene to dairy cows at a rate of 110 g/kg diet DM, and found that neither a 215 bp segment of the transgene nor a 400 bp segment from the endogenous *acyl carrier protein* gene was amplifiable from milk. Other studies investigating the fate of transgenes and low copy endogenous genes in milk from animals fed GM cotton (Jennings et al., 2003c), maize (Phipps et al., 2003; Jennings et al., 2003c; Yonemochi et al., 2003), soybean (Phipps et al., 2002) or non-GM maize and

soybean (Poms et al., 2003) have yielded negative detection results, suggesting that while absorption of plant DNA is possible throughout the ruminant digestive tract, passage of foreign plant low-copy DNA into milk is unlikely.

5.4. Pigs

Chowdhury et al. (2003c) analyzed transgenic and intrinsic DNA fragments by PCR throughout the intestinal tract of pigs fed diets containing 700 g insect-resistant maize/kg. Primer sets detected two fragments of the endogenous *zein* gene (242 and 329 bp) and two fragments of the transgenic construct that encoded the *cry9C* gene (103 and 170 bp). None of the plant sequences was detected in the duodenal contents. However, this may have resulted from feed passage to the large intestine, as sampling of digestive contents took place the day after the last feeding. Both endogenous and transgenic DNA fragments were detectable in cecal and rectal contents of pigs at frequencies ranging from 0.25 to 0.50, indicating that plant DNA persists throughout the porcine GIT when such a diet is consumed. Another study detected 3 separate endogenous genes and 2 transgenic fragments of varying size from digestive contents of the stomach, duodenum, ileum, cecum, and rectum of 10 pigs fed diets containing 600 g insect-resistant maize/kg (Chowdhury et al., 2003b). All of the endogenous fragments, which included sequences from the *rubisco* (1028 bp), *invertase* (226 bp), and *zein* (242 bp) genes were detected in 0.3–1.0 of the samples, depending on the origin of the contents and primer set. The relatively large amplicon size of the *rubisco* gene indicates that even substantially sizeable fragments of DNA survive the digestive process in pigs. However, the authors noted that maize kernels were visible within gastrointestinal contents. It is therefore possible that DNA detected near the end of the digestive tract was protected within undigested maize residue that was inaccessible to DNA-degrading enzymes. Unlike ruminant studies, the relative stability of free DNA in pig digestive contents has not been reported. The two transgenic fragments (110 and 437 bp) were located within a construct containing the *cry1Ab* gene. Each sequence was detected to the same extent in the stomach (1.0), however the larger fragment was detected less frequently than the small fragment in duodenal (1.0 *versus* 0.60), ileal (1.0 *versus* 0.60), cecal (1.0 *versus* 0.60) and rectal contents (0.60 *versus* 0.20). This does not necessarily infer that the smaller fragment was more persistent than the larger one, as LOD, which was not reported, could account for differences in sensitivity. Generally, smaller amplicons result in greater PCR efficiency. In theory, if undigested plant residues (*i.e.*, undigested plant cells) are present in a digestive sample, then all parts of the genome should be amplifiable. Whether plant DNA persists in a free form or is feed-associated throughout the pig digestive tract remains to be investigated.

Klotz et al. (2002) attempted to describe the time-dependent persistence of plant DNA in the upper part of the GIT of pigs. Pigs maintained on a non-transgenic diet were fed 1 kg of a ration comprising 0.500 insect-resistant maize (event 176), after which they were slaughtered at intervals over the next 12 h. A 199 bp sequence of plant chloroplast DNA was amplifiable from contents of the stomach, duodenum, jejunum, and ileum for 12 h after feeding, although the intensity of each PCR product eventually diminished over time. In contrast, transgenic sequences of the *cry1Ab* gene (211 and 251 bp) were not detected from any digestive sample at any time point. The differences are likely due to the number of copies of each gene per genome. Chloroplast DNA genes can be present at between 500 and

50,000 copies (Bendich, 1987) compared to the single insert of most transgenes. In addition, neither the endogenous nor the transgenic DNA sequences could be detected in blood or lymph nodes from the animals at any time. In contrast, Reuter and Aulrich (2003) detected the transgene fragments in digesta from pigs fed Bt176 maize. In that study, pigs were fed 2.6 kg/d of a ground diet (1 mm particle size) containing 700 g maize/kg throughout the fattening phase. A 211 bp fragment of DNA amplified from the *cry1Ab* gene was detected in the stomach up to 24 h, the duodenum, jejunum and ileum up to 48 h, the cecum up to 12 h, colon up to 24 h, and rectum up to 48 h after feeding the diet. A 140 bp fragment from chloroplast DNA was detected in every type of sample taken from the GIT, even 72 h after feeding. While it might be expected that DNA digestibility would increase with the degree of grinding of the feed, the amount of transgenic DNA ingested will also affect the likelihood of gene persistence within the digestive tract. This may explain the differences in results between the aforementioned studies. Klotz et al. (2002) fed the pigs 0.5 kg of insect-resistant maize whereas the animals in the study by Reuter and Aulrich (2003) were fed 1.82 kg of maize per day.

The above two studies also attempted to detect plant DNA in animal tissues. Reuter and Aulrich (2003) were able to detect the 140 bp fragment for chloroplast DNA in blood, liver, lymphatic glands, spleen, kidney, *musculus gluteus maximus*, *musculus*, *longissimus dorsi*, *musculus trapezius* and ovary samples in 0.167, 0.54, 0.167, 0.125, 0.27, 0.333, 0.542, 0.229, and 0.625 of the samples tested, respectively. Again emphasizing the importance of transgene copy number in digesta detection, the transgenic DNA from the single copy *cry1Ab* gene was never detected in any sample. The relatively high detection rate in the ovaries was suggested to result from high blood flow to that organ. Klotz et al. (2002) were also unable to detect transgenic DNA in muscle, liver, spleen, lymph nodes and blood from pigs fed diets containing 200–250 g maize/kg. In addition, and again in contrast to Reuter and Aulrich (2003), chloroplast DNA was also undetectable in any of the mentioned tissues. These differences between and within each study likely highlight the significance of the number of genes ingested, or perhaps the sensitivity of methods employed, since the diet fed by Reuter and Aulrich (2003) was finely ground thus particle size was unlikely a factor in gene persistence. The animals in the study by Klotz et al. (2002) consumed fewer copies of transgenic and endogenous maize genes than were fed by Reuter and Aulrich (2003), as mentioned above. Moreover, the amounts of the chloroplast sequences targeted in detection of plant-specific chloroplast DNA may have differed at the genome level. Consequently, intake could have been substantially different between experiments, as the copy numbers of the chloroplast targets were not reported. Additionally, the intake of transgene compared to chloroplast gene is low which may result in differential persistence and detection between the two genes.

Another factor that will directly affect gene persistence throughout the digestive tract and therefore indirectly affect the chance of passage across the GIT epithelium is the digestibility of the ingested plant species. Feedstuffs with relatively greater digestibility, such as soybean meal, are likely to have their DNA degraded more rapidly, decreasing the chance for absorption. Jennings et al. (2003a) attempted to detect a low copy endogenous and recombinant gene in *longissimus* muscle samples from pigs fed herbicide-tolerant or conventional soybean meal at 240 to 140 g/kg diet, in the grower and finisher phases, respectively. The study PCR followed by Southern hybridization to create highly sensitive test assays for

sequences of the intrinsic *lectin* gene (198 bp, LOD = one diploid genome equivalent) and recombinant *cp4 epsps* gene (272 bp, LOD = one diploid genome equivalent). None of the samples tested positive for either gene.

The presence of plant DNA in 118 samples from the *longissimus* muscles of pigs fed insect-resistant maize (event MON810) or an isogenic control was explored by Nemeth et al. (2004). They used highly sensitive primers, as indicted by low LOD, that amplified two fragment lengths from a chloroplast *rubisco* gene (173 bp, LOD = 0.02 genome equivalents; 500 bp, LOD = 0.08 genome equivalents) and a fragment from the CaMV promotor gene (*P-35S*; 123 bp, LOD = 5 genome equivalents) that is the promoter of the transgenic construct. If the muscle samples that tested positive for *P-35S*, analysis for MON810 construct-specific sequence (149 bp, LOD = genome equivalents) was carried out. For the 173 bp *rubisco* sequence, 0.53 of the samples tested positive (both duplicates positive), 0.43 negative (both duplicates negative) and 0.04 indeterminate (duplicate samples comprised one positive and one negative). The 500 bp *rubisco* fragment was present in 0.43 of the samples, undetected in 0.43 and indeterminate in 0.14. One tissue sample out of the total 118 tested positive for the *P-35S* sequence. To confirm the results, the analysis was repeated with new tissue sub-samples, which again were positive. However, when tested with the MON810 primer set, the result was indeterminate. This suggests that the number of transgene copies was below the LOD. The actual rate of occurrence of such an event (*i.e.*, uptake of transgene) is likely so low that an extremely large sample population would be required to demonstrate statistical significance. The principal conclusion from these findings is that transgenic DNA acts similarly to endogenous DNA, *i.e.*, that if present in enough quantity, it may cross the gastrointestinal barrier. Additionally, despite detection of the transgene in pork tissue, there was no difference in the growth performance or meat quality between pigs fed diets containing transgenic or conventional maize (Weber et al., 2000). Thus, neither transformation of the maize nor the transgene itself adversely affected animal health.

5.5. Poultry

Chambers et al. (2002) investigated the fate of plant DNA throughout the digestive tract of chickens fed insect-resistant maize (event 176) present at 800 g/kg diet. PCR-restriction fragment length polymorphism (RFLP) analysis indicated that the *beta lactamase* (*bla*) gene, present as part of the transgenic construct found in event 176 maize, could be detected in the crop of each bird tested ($N = 5$) and in the stomach of two of the birds. Results for the transgenic DNA were negative in the small intestine, large intestine, cecum, and rectum. Detection of the maize mitochondrial gene, *nad5*, followed a similar pattern, being present in the crop and stomach of all birds. Mitochondrial genes are generally present at a higher copy number than are nuclear encoded genes, which likely accounts for the higher rate of detection from digesta contents of these two segments. The *nad5* gene was also not detected in any of the other digestive contents. A study testing diets containing the same insect-resistant maize at 600 g/kg yielded confirmatory results (Aeschbacher et al., 2005). The transgenic *bla* (479 bp) could be detected in the crop of broilers by PCR and not in the gizzard, small intestine, cecum, and excreta. The endogenous *invertase* gene was also limited mostly to the upper part of the digestive tract, being detected in the crop, gizzard, and to a lesser extent, the small intestine. Contradicting the above two studies was an

experiment that detected DNA throughout the GIT of poultry when the same Bt176 maize was fed at 740 g/kg (Tony et al., 2003). Using real time PCR, the authors showed that sequences of the maize-specific *hmg* gene (79 bp) and the transgenic *cry1Ab* gene (129 bp) were detected in the crop, proventriculus, gizzard, duodenum, jejunum, ileum, cecum and rectum. Although quantitative data were not presented, the threshold cycle was reported for each of the digesta contents up to 24 h after withdrawal of feed. The threshold cycle is inversely proportional to the quantity of amplicon in a sample. The pattern of detection of *hmg* was similar between birds fed transgenic maize and those fed an isogenic control and was also similar to that of the *cry1Ab* gene in birds fed transgenic maize. For the most part, the threshold cycle for both amplicons generally increased with increasing post-feeding interval. This likely reflects a combination of passage of the genes throughout the GIT and their degradation. The authors suggested that maize DNA can persist throughout the GIT without much degradation and is excreted in the fecal matter. This may be misleading however, when the threshold values are considered. In some cases, the threshold values increased from about 25 at 0 h to approximately 38 at 24 h. Theoretically, each increase or decrease in threshold value represents a two-fold change in gene copy, assuming an efficient PCR. Therefore, a threshold difference of 13 units reflects a 4096-fold change in concentration. This implies significant degradation or passage over time. However, the differences in persistence between the studies may also reflect smaller PCR products in the latter experiment.

Using conventional PCR, Tony et al. (2003) were unable to detect either a 211 bp sequence of transgenic DNA or a 226 bp sequence of the endogenous *invertase* gene in blood, pectoral, thigh, liver, heart, spleen, kidney, bursa, or thymus tissues from broilers fed diets containing 736 g Bt176 maize/kg. Like the transgene, the *invertase* gene is a low copy gene and is present at one copy per plant genome (Hernandez et al., 2004). However, a 199 bp sequence of high copy chloroplast DNA was detected in all these tissues except the heart, bursa, and kidney, up to 4 h after feed withdrawal. Aeschbacher et al. (2005) reported similar results for transgenic DNA detection for birds fed diets containing 600 g Bt176 maize/kg. The transgenic *bla* gene (479 bp fragment) was not identified in the liver, spleen, muscle, blood, or eggs of hens or broilers but was faintly identifiable in the crop of broilers. Those authors detected the same low copy 226 bp *invertase* sequence that Tony et al. (2003) used, in the liver, spleen, muscle, blood, crop, gizzard, and small intestine of broilers but not in the cecum or excreta. Evidence suggests that absorption behaviour of DNA is uniform across sources, thus, it would be expected that the absorption of one low copy gene fragment should signify the absorption of other low copy gene fragments, including those from the transgenic *bla* gene. However, fragment absorption may be size-dependent and the authors did note that the majority of DNA recovered from the digestive tracts of the birds were less than 180 bp, smaller than the amplicon size of the *bla* primer set.

Klotz et al. (2002) also attempted to detect plant DNA in poultry tissues and provided evidence of a size-dependent effect on fragment absorption. Commercial market samples of breast, leg, stomach, and wing tissues from turkeys and chickens were tested for fragments of endogenous maize genes, including a 532 bp fragment of chloroplast DNA, a 199 bp fragment of chloroplast DNA, and a 277 bp fragment of the *zein* gene. None of the samples was positive for the 532 bp chloroplast sequence, however the 277 bp fragment was detected

in all of the samples. Although the LOD was not reported for the primer sets, it is probable that shorter DNA fragments more readily cross the intestinal barrier. The *zein* gene, which is a low copy gene present at roughly two copies in the haploid maize genome (Hernandez et al., 2004) was also faintly detectable in the samples. The authors did not test for transgenic DNA but from this study and that by Tony et al. (2003), it appears that short fragments of low copy plant genes have the potential to be absorbed throughout the avian digestive tract and incorporated into body tissues. In contrast, the DNA fragments were not detected in the embryos of eggs. Einspanier et al., 2001, were also unable to detect the same 199 bp chloroplast fragment in the eggs from chickens fed diets containing 500 g Bt 176 maize/kg. These studies together suggest that plant DNA is less likely to cross into the eggs than to be incorporated into body tissues of chickens. The fragment was amplified in muscle, liver, spleen, and kidney tissues. However, a 189 bp segment of transgenic DNA was not detectable in any of the samples.

Two studies have also tested for transgenic DNA in tissues of chickens fed diets containing insect-resistant MON810 maize at approximately 600 g/kg (Jennings et al., 2003b; Nemeth et al., 2004). Jennings et al. (2004) again used a PCR-Southern hybridization method that enabled detection of one copy of the diploid genome with primer sets for sequences from endogenous *sh2* (213 bp) and transgenic *cry1Ab* (211 bp). Both genes are present at low copy numbers, but despite adding 1 μg of extracted muscle DNA to the PCR reactions, neither gene sequence was detected in any of the samples. Nemeth et al. (2004) were unable to detect the 149 bp segment of the transgenic construct in breast muscle, however a 173 bp sequence of the high copy chloroplast *rubisco* gene was positive in 0.15 of the samples, negative in 0.75 of the samples, and indeterminate in 0.10 of the samples, again emphasizing the critical influence of gene copy number on the sensitivity of detection.

6. Conclusion

Agriculture has evolved to produce and select plants with more desirable traits. Modern recombinant DNA technology has allowed for the introduction of DNA from any source into plant species. Globally, the area seeded to GM crops increased to 81.0 million ha in 2004. Detection of GM products is performed by protein and DNA assays. Both of these methods are capable of detecting and quantifying transgenic plants below the thresholds set by most countries. However, because after processing or combination in mixed diets feeds can represent complex matrices, validation studies are necessary to provide accurate detection and quantification of any GM content in feeds destined for countries with labeling laws pertaining to feeds. To ensure the safety of GM plants as animal feed, regulatory bodies have adopted the policy of substantial equivalence. Though this does not prove nutritional equivalence, to date there have been no adverse effects in animals consuming commercialized GM crops approved on the basis of substantial equivalence. Studies undertaken to address concerns that transgenic protein or DNA may enter the market by means of animal products have shown that recombinant materials in transgenic feed are unlikely to be incorporated into animal products at significant levels. Absorption of plant DNA across the intestinal barrier in food animals does seem to be a normal occurrence when DNA fragments are present at high concentrations. Absorption of DNA does not appear to have adverse effects

on livestock, whether the DNA is transgenic or endogenous. Given the popularity of GM crops, which is expected to increase over the next few years, GM plants in the food-chain should be monitored continually. Rigorous testing procedures for novel crops should remain in place, especially when traits are introduced that affect nutritional components. In these instances, nutritional equivalence should be determined by performing animal trials, in addition to substantial equivalence tests. However, the fate of recombinant molecules in the currently registered GM plants need not be included in feed safety assessments.

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