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**Submission on APPLICATION A549 FOOD DERIVED
FROM HIGH LYSINE CORN LY038: to permit the use in
food of high lysine corn**

Submitted to Food Standards Australia/New Zealand (FSANZ)

by

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Overview

This submission from the New Zealand Institute of Gene Ecology (NZIGE) is meant to support Food Standards Australia/New Zealand's preparation of a Draft Assessment on application A549. Our comments and wording are direct, but our spirit is constructive. The NZIGE is dedicated to the development for the public good of all responsible biotechnologies. We are an assemblage of serious researchers with independent credentials in the area of biotechnology and its social impact. The activities of the NZIGE were supported in part by a grant for the Biosafety Forecast Service of the Biosafety Capacity Building Package under the auspices of the Norwegian Institute of Gene Ecology (GENØK).

A549 is an application to amend the Australia New Zealand Food Standards Code to allow foods derived from corn line LY038 to be sold in Australia and New Zealand. "Corn line LY038 has been genetically modified to have higher than usual levels of the amino acid lysine," particularly in the corn grain.¹ LY038 was modified by the gene *cordapA*, sourced from the bacterium *Corynebacterium glutamicum*, inserted into the corn genome using genetic engineering techniques. The gene "encodes the enzyme dihydrodipicolinate synthase (DHDPS). This enzyme is involved in lysine biosynthesis. The bacterial DHDPS enzyme, unlike the plant DHDPS enzyme, is not sensitive to lysine feedback inhibition, so lysine biosynthesis will continue in the presence of high levels of free lysine."²

Our submission begins with introductory material describing who we are and why we are involved. We then provide a summary of the major recommendations gathered from the detailed sections of our submission. These sections are organized into three main parts. In Part One, we undertake risk *forecasting*, an exercise at the leading edge of the research literature that serves to forewarn of risk where the science is not certain. Novel potential hazards of *C. glutamicum* Dihydrodipicolinate Synthase (cDHDPS) protein, its metabolic products expressed in maize, and other side-effects of inserting DNA into the maize genome were identified to the best of our ability on the very tight timeframe available to us for this phase of consultation. Some of these properties, moreover, will be particularly influenced by the protein's environment and thus are even more important for assessments of food safety.

In Part Two, we review the scientific documents submitted by the Applicant in support of A549. We judged this material by two criteria: 1. Was the science at the best possible standard? and 2. Does the science add up to a package that is sufficient to assure the citizens of Australia and New Zealand that they may safely consume food derived from corn line LY038? In most cases we recommend how, why and when the Applicant should supplement their findings with additional data.

In Part Three, we comment upon the Impact Analysis contained in the Initial Assessment Report (IAR). We assess the costs and benefits listed and propose further costs and benefits

¹FSANZ (2004). Initial Assessment Report: Application A549 Food Derived from High Lysine Corn LY038, p. 6.

²Ibid, p. 9.

of the options under consideration.

The Authority (FSANZ) has made plain “the need for standards to be based on risk analysis using the best available scientific evidence”³. Above this need is the objective of the “protection of public health and safety” and “the provision of adequate information relating to food to enable consumers to make informed choices”⁴, which requires the Authority to determine if the best scientific evidence available is good enough. Our contribution has therefore been to help the Authority identify areas of scientific uncertainty in the application so that these uncertainties can be addressed during the Authority’s development of a complete assessment.

We provide compelling new scientific evidence of risk and hazard. We also cannot exclude certain hazards from the information in the studies submitted by the Applicant and made available to the public by FSANZ.

- *The transgenic protein cDHDPS may have a different risk spectrum when a component of food.*
- *cDHDPS and its catabolic products could create novel risks in processed or cooked food.*
- *The creation of novel RNA molecules by insertion of DNA into the maize genome could create species of RNA that are harmful to humans, possibly through food.*
- *The molecular characterization of the DNA inserted into the maize genome, the LY038 event, and DNA donated from the transgenic Cre-recombinase line used to create the LY038 maize line, is incomplete. The present data does not exclude, with a high level of confidence, the possibility that corn line LY038 contains additional novel genes, be they derived from the expression of fragments of inserted DNA or novel fusion proteins created at the junctions of inserted DNA and the maize genome.*
- *The molecular characterization of the transgenic protein cDHDPS produced by the genetically modified plant is flawed because the Applicant has not demonstrated that all novel proteins were included in this analysis.*
- *The digestibility study of cDHDPS, required as part of an assessment of allergenicity, does not meet FAO/WHO standards for concentration of pepsin or standard comparisons to known allergens. Moreover, the digestibility study was fundamentally flawed by not using material from the actual genetically modified organism that the people of Australia and New Zealand would be eating.*
- *An adequate molecular characterization of all novel RNA molecules, that may pose a risk to consumers, is missing along with microarray analysis of the transcriptome of the LY038 line. There is published evidence that genetic components of the LY038 event produce novel RNA molecules. There is also evidence in animal studies that some small RNA molecules can be transmitted through food, causing lasting, sometimes heritable, effects on consumers and their*

³FSANZ (2004). Initial Assessment Report: Application A549 Food Derived from High Lysine Corn LY038, p. 9.

⁴Ibid, pp. 8-9.

children.

- *The data comparing the composition of the transgenic lines to commercial reference lines of maize may be skewed by selective choice of commercial lines. The commercial reference lines chosen may inflate the 99% tolerance interval to more closely match the composition of LY038, thereby reducing the apparent number of significant compositional differences between the LY038 line and conventional corn.*
- *The compositional analysis does not appear to fully support the conclusion of equivalence between LY038 and its closest relative. The comparison found 103 (26% of total comparisons across 5 field studies) statistically significant differences between LY038 and the negative segregant.*
- *The acute toxicity study was fundamentally flawed by not using material from the actual genetically modified organism that the people of Australia and New Zealand would be eating.*
- *The broiler performance study may have falsely overestimated the positive effects of LY038 on chickens due to the choice of commercial reference controls.*
- *The broiler performance study indicates some unexplained negative effect on growth over the first 21 days when broilers were fed LY038.*
- *A549 lacks a subchronic toxicity study of adequate duration to conclude that the amino acid levels in LY038 are safe.*
- *A549 lacks a long-term toxicity and carcinogenicity study necessary to conclude that the amino acid levels in LY038 are safe.*

We also provide information and analysis indicating that the Impact Analysis is currently incomplete in some respects and mistaken in others. Addressing these deficiencies would significantly shift the balance of the analysis.

We encourage a precautionary approach when assessing LY038. The scientific community is not uniformly convinced about the adequacy of existing risk assessments (Pusztai, et al. 2003), comfortable with the evidence that genetically modified food organisms are generally safe (Pryme and Lembcke 2003), nor confident that if approval were revoked, a GMO could be removed from the food chain before it caused harm (Heinemann, et al. 2004).

FSANZ has stated that the primary data⁵ received from Applicants in support of their claims “enables a more rigorous analysis of experimental outcomes than the summary data of the type submitted in support of publication of a scientific article in a peer reviewed journal.” On the contrary, the data we have seen in A549 is not so different from that included in papers we have reviewed for journals. Nevertheless, direct access to the primary data is certainly an important requirement. It is important to note that, just as when peer-reviewing papers for publication, the reviewer cannot ‘tweak’ the experiment or

⁵Not ‘raw’ data as indicated in the FSANZ document “FSANZ Response to Article Entitled ‘GE Foods and Human Health Safety Assessments’ By Dr Judy Carman, Spokesperson on GE Food, Public Health Association of Australia”, unless FSANZ receives machine print-outs and traces as well as photographs and tables.

explore all the unwritten parameters. This can lead to mistakes in reviewing. And while the publication of a paper with a flaw generally has very little influence on the daily lives of most citizens, the change in the New Zealand and Australia Food Code has implications for tens of millions of people directly and, because it may be connected to changes in global agriculture, it could have global ramifications. Therefore, the standard of review must both be better and more interrogating than for routine research results submitted for publication.

We have the view that truly good biotechnologies will be vindicated by not just the best available science, but science adequate to the task of making a sound decision on safety. Our *a priori* view is this: it is not a given that the science of the day is adequate for the task. It is possible for an applicant to do state-of-the-art analyses and not meet a standard of risk identification or resolution that may be necessary.

Should the best available science be ambiguous on A549, then New Zealand's precautionary stance (as defined by the Convention on Biodiversity and the Hazardous Substances and New Organisms Act 1996 and amendments) must take priority.

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Abstract

In the IAR, FSANZ invited “individuals and organizations to assist FSANZ in preparing the Draft Assessment for” this application A549 (FSANZ 2004, p. 3). Significant new evidence and analysis that is specifically relevant to the evaluation of LY038 is provided to support FSANZ in their preparation of a Draft Assessment. Our submission relates to “the scientific aspects of this Application, in particular, information relevant to the safety assessment of food from corn line LY038” (FSANZ 2004, p. 7). It also addresses the consistency of the IAR with “the objectives of FSANZ as set out in section 10 of the FSANZ Act” and provides “details of potential costs and benefits of the proposed change to the Code” (FSANZ 2004, p. 3).

In Part One, we describe important structural differences between mDHDPS and cDHDPS that produce novel challenges for studies on allergenicity, developments in food safety science regarding novel aggregates that could be produced by transgenic cDHDPS, the importance of characterizing post-processing and cooking effects on lysine, lysine catabolites and cDHDPS itself, novel regulatory RNAs that may have physiological effects on human consumers and species of DNA that could have biological effects if taken up by human cells through food.

In Part Two, we provide the Authority with a detailed examination of the molecular biological data supplied by the Applicant. We find suggestions in the Applicant’s data that secondary insertions into the LY038 genome may have been overlooked, and find deficiencies in protein identification protocols that could lead to false confidence in the low number of novel proteins and post-translational modifications reported in A549. Most importantly, new evidence suggests that the nos terminator sequence used in LY038 is a recombination hotspot, prone to read-through and may contain a cryptic *cis*-acting splice sequence that could generate novel RNA molecules and proteins at any place it is inserted into the genome. The lack of holistic proteomic and microarray analysis is a serious deficiency of this application.

In Part Three, we evaluate the cost/benefit analysis conducted by the Authority. One of our chief concerns with this analysis is that the costs and benefits included are largely concerned with *feed* while the Authority is considering whether to amend the *food* Code. Only when the place of feed-related impacts in a food assessment is clarified can one accurately assess the costs and benefits of Options 1 and 2. We also note that significant costs and benefits have been omitted from the analysis, and evidence is lacking to support those that have been included. In addition, the premises underlying some of the listed costs and benefits require further scrutiny.

Our conclusion is that significant additional information should be provided by the Applicant before the Australia New Zealand Food Code is amended. There have been important advances in biosafety and risk assessment science that are not uniformly reflected in the standard of reporting in A549. The studies submitted in support of A549 no longer uniformly meet what we see as the standard of the science.

We have raised *bona fide* issues of safety that have not been addressed by the Authority or the Applicant, which also increases the cost of amending the Code. We have not found in the economic analysis any evidence of how amending Standard 1.5.2 will directly reduce food costs. In fact, we see no evidence that the consumer stands to receive any *food* benefit from amending the Code, while some consumers will certainly bear additional costs from doing so. We therefore must reject most of the Authority's speculation on benefits in opting for Option 2.

Should the Authority come to recommend that the Code be amended to include event LY038, then it is our opinion that a special condition be imposed upon event LY038. In Column 2 of the Table to Clause 2 of Standard 1.5.2, LY038 but not any other members of the LY038 line currently in existence or that may arise through breeding, hybridization or transformation in the future may benefit from a favorable assessment of LY038. In other words, approval of LY038 does not extend to other lines of maize that share parentage or the I-DNA with LY038.

I. Introduction

- I.1 This submission is the opinion of the submitter on Application A549 – high lysine corn LY038 ‘MAVERA HVC with Lysine’.
- I.2 The submitter is the New Zealand Institute of Gene Ecology (NZIGE) and its cooperating partners. Responsibility for the content of this submission rests solely with the authors and NZIGE. The NZIGE is a research organisation (www.nzige.canterbury.ac.nz). The NZIGE has no commercial interest in the product at the focus of this application, no direct or indirect connections with the Applicant, and has no connections to parties that seek to compete with the Applicant by developing a similar novel food. We received substantial support for the research that informs this application through a grant from GENØK for the Biosafety Forecast Service, and additional support from the University of Canterbury. Our submission is further informed by our own extensive experience in the research areas discussed below. If there were to be a public hearing on the application, we would be pleased to present our view.
- I.3 Our submission relates to “the scientific aspects of this Application, in particular, information relevant to the safety assessment of food from corn line LY038” (FSANZ 2004, p. 7). It also addresses the consistency of the IAR with “the objectives of FSANZ as set out in section 10 of the FSANZ Act” and provides “details of potential costs and benefits of the proposed change to the Code” (FSANZ 2004, p. 3).
- I.4 We have done our best to evaluate the scientific documents supplied by the Applicant in support of the application. Some areas of uncertainty may have arisen from the poor reproduction of some material made available by FSANZ for our use.

R. Summary of major recommendations

- R.1 In order to make an assessment of the changes in protein expression that occur within the plant due to expression of cDHDPS, the Applicant should undertake a routine proteomic analysis (by comparative 2D electrophoresis and mass spectrophotometric analysis of relevant spots) of lysates from whole plant cells and demonstrate that the only change is expression of the inserted gene. Single dimension protein gels of whole plant extracts at various stages of purification should also be supplied, in order to authenticate the purification of cDHDPS from plant tissue. Presentation of only the purified protein is unacceptable.
- R.2 The Authority should require data from long term (lifetime) animal feeding trials to capture chronic effects, detect carcinogens and co-carcinogens, and proteins that are capable of forming amyloid fibrils.
- R.3 The Authority should request an analysis of Maillard reaction products or other glycotoxins that could arise from cooking or processing of LY038 corn.
- R.4 The Applicant should test the potential of *in planta*-produced cDHDPS to form amyloid fibrils and measure the cytotoxicity of aggregates and intermediate forms

compared with native cDHDPS. It would be highly desirable to have the aggregation potential of cDHDPS correlated with changes in pH and other varying physical parameters of the chloroplast.

- R.5 In order to make an assessment of global changes in the transcriptome, and specific changes cause by the insertion(s) of I-DNA, the Authority should require microarray descriptions capable of detecting novel RNA species in the modified plant, with the RNA source being the plant grown under a variety of relevant field conditions. The microarray should comprehensively represent the genomes of the cultivar of maize modified and unmodified, and any novel RNA species should be tested against the human genome for RNAi activity.
- R.6 We recommend that I-DNA, especially the G1b1 promoter sequence, be analysed for putative mammalian transcription factor binding motifs.
- R.7 We recommend that a metabolomic analysis such as NMR combined with chemometrics and univariate statistics be supplied to the Authority by the Applicant.
- R.8 The Authority should require the Applicant, at a minimum, to supply data on the digestibility of the cDHDPS protein using a protocol consistent with the FAO/WHO standard (FAO/WHO 2001) and the recommendations of Pusztai *et al.* (Pusztai, et al. 2003).
- R.9 We recommend that a compositional analysis that includes the four commercial varieties used in MSL-18883 be requested by the Authority.
- R.10 We recommend that the Authority dismiss study MSL-18883 for purposes of assessing safety.
- R.11 The Authority should require the Applicant to submit data on cholesterol concentrations in serum and lipoprotein, and any changes in liver phospholipids, in animal feeding experiments.
- R.12 We recommend that the Authority request the Applicant to provide a valid subchronic toxicity study of a minimum of 6 months duration.
- R.13 We recommend that only after the Applicant demonstrates the safety of LY038 using a higher standard of *in vitro* and animal *in vivo* safety tests, then human tests should be completed before another application is lodged with the FSANZ.
- R.14 A plan for effective post-launch monitoring should be provided by the Applicant and the plan should be subject to a transparent review through the independent scientific community.
- R.15 The Authority should clarify its proper jurisdiction with regard to this Application; in particular, it should clarify whether and how it is equipped to analyse the impact of the availability or non-availability of LY038 animal feed.
- R.16 We urge the Authority to disregard the declared intention of the Applicant to segregate LY038 from the human food supply, as it cannot bind the Applicant (or

any other party) to this action once the Food Code has been amended. We recommend that the Authority ensure that this declared intention not be permitted to influence the rigour of the application or the analysis of its impacts.

- R.17 The Authority should clarify the reasoning behind its identification of Affected Parties; in particular, it should clarify why failing to amend the Food Code would prevent animal growers accessing LY038 *feed*.
- R.18 The Authority or Applicant should provide evidence for its assertion that the cost to consumers of avoiding LY038, and to government of monitoring for the presence of LY038 in food, will be low.
- R.19 The Authority should not extend any approval of LY038 to any hybrid line derived from LY038. As in European regulation, all hybrids, whether between LY038 and an unmodified line or another approved modified line, must in this case be treated as a new organism requiring a full safety evaluation.
- R.20 The Authority should take into account the implications of approving this amendment of the Food Code for New Zealand's obligations under the Cartagena Protocol.

Part One: Risk forecasting

(1) Potential proteome, transcriptome and genomic changes

1.1 Structure and biochemistry of prokaryotic vs. eukaryotic DHDPS

- 1.1.1. Whilst there is no doubt that the bacterial and plant enzymes share a common biochemical function (albeit with altered regulatory patterns) and perform identical chemistry *in vitro*, there are important differences between the two types of enzyme that do not seem to have been appreciated by the Applicant.
- 1.1.2. The *C. glutamicum* enzyme (cDHDPS) shows high similarity to the *Escherichia coli* enzyme, the structure of which has been solved (Mirwaldt, et al. 1995). Similarly, the maize enzyme shows a high similarity to the only plant enzyme, that from *Nicotiana sylvestris*, for which the structure is known (Blickling, et al. 1997). Both prokaryotic DHDPS and eukaryotic DHDPS are tetrameric and have a common fold in their tertiary structure.
- 1.1.3. Natural DHDPS is a highly unusual enzyme, however, in that the bacterial and plant enzymes have an entirely *different quaternary structure* due to a profound rearrangement of the dimers forming the tetramer. This difference in molecular architecture means that different faces of the protein are presented to the solution.
- 1.1.4. The key concern of this observation is that the epitopes of mDHDPS and *in planta*-produced cDHDPS could be very different and the potential allergenicity of the transgene may be different from expected.
- 1.1.5. New allergens in maize could be especially problematic for some groups of people. This highlights one of the social implications of commercial GMOs. There is an increasing awareness in the medical community of the allergenicity of wheat proteins to a high portion of the Western population, resulting in coeliac disease (sprue) and related diseases associated with gluten intolerance. Some studies estimate that 1 in 300 people are coeliac, with a much higher number suffering some form of gluten intolerance (Kennedy and Feighery 2000). Such individuals rely heavily on maize as one of the few commercial grain crops that is safe to eat, and may have particular concerns about the potential allergenicity of proteins derived from transgenes especially because they might further reduce food options.

1.2 Potential differences in the tendency of maize DHDPS (mDHDPS) and cDHDPS and their degradation products to form toxic aggregating species (e.g. amyloid fibril precursors).

- 1.2.1. “Aggregation is a general feature of proteins and can be classified into two types: *in vivo* aggregation such as inclusion body formation and *in vitro* aggregation such as denaturation-induced aggregation. Amyloid fibril formation is an *in vivo* phenomenon, but it also occurs under artificial conditions *in vitro*” (Ohnishi and Takano 2004, p. 517). The aggregation to which we refer is ordered aggregation or amyloid fibrils (Fernandez-Escamilla, et al. 2004).

- 1.2.2. Protein misfolding and subsequent aggregation into amyloids are hallmarks of a group of diseases termed “amyloidoses”. While the toxic form of the protein is still debated, it is most likely an intermediate between the native and fibril form of the protein (Bucciantini, et al. 2002).
 - 1.2.2.1 Amyloid fibrils are also found in neurodegenerative diseases such as Parkinson’s disease (PD), Huntington’s disease (HD), and the prion diseases (transmissible spongiform encephalopathies, TSEs) (Dobson 2001). Other non-neurological amyloidoses include hypercholesterolaemia, type II diabetes and primary and secondary systemic amyloidoses (Chiti, et al. 2000, Dobson 2001, Pepys 2001). “There are also various local forms of amyloidosis in which deposits are confined to specific foci or to a particular organ or tissue. These may be clinically silent or trivial, or they may be associated with serious disease”(Pepys 2001, p. 203).
 - 1.2.2.2 Probably all proteins can form amyloid fibrils under conditions encountered in some intracellular environments, as mild denaturing conditions that *in vitro* conditions mimic, or if primary structure favours re-folding (Chiti, et al. 2000). The intracellular compartments within lysosomes, the ER and proteasomes may be especially conducive to refolding.
 - 1.2.2.3 Although each of the known amyloidogenic proteins has a distinct function and shows no significant similarity in sequence and native structure to any of the other proteins of that family, they all adopt a very similar conformation when aggregated in amyloid deposits.
- 1.2.3. Even normally benign proteins can produce cytotoxins as they transit from partially to fully unfolded states and eventually form amyloid fibrils (Ellis and Pinheiro 2002, Fernandez-Escamilla, et al. 2004) although some evidence suggests that granular aggregates can also be toxic (Bucciantini, et al. 2002).
 - 1.2.3.1 “The data, therefore, suggest that impairment of cell viability by protein aggregates of the type that can subsequently form amyloid fibrils could be a general phenomenon and not simply a specific property of the small number of polypeptides associated with clinically recognized protein deposition diseases. This result is of particular significance in the light of the recent conclusion that the ability to form highly ordered amyloid fibrils is itself a generic property of proteins” (Bucciantini, et al. 2002, p. 509).
- 1.2.4. Amyloid fibrils may or may not be harmful to the organism in which they arise, or the harm may not be apparent immediately, as in Mad Cow Disease and variant Creutzfeldt-Jakob Disease (vCJD). Still, they may be transmitted through food and are therefore a food safety issue.
- 1.2.5. The Applicant should test the potential of *in planta*-produced cDHDPS to form amyloid fibrils and use an *in vitro* human tissue culture assay to measure the cytotoxicity of aggregates and intermediate forms compared with native cDHDPS. It would be highly desirable to have the aggregation potential of cDHDPS correlated with changes in pH and other varying physical parameters of the chloroplast.

- 1.2.6. We would not expect potentially dangerous aggregates to be detected in either the broiler feeding study or the acute mouse toxicity study, because exposure to some aggregated proteins in the amyloid form can take decades to produce an effect. This is a reason that the Authority should insist on long term (lifetime) animal feeding trials.
- 1.3 Creation of novel regulatory RNA molecules that also may be transmissible through food.
 - 1.3.1. RNA regulatory molecules are best known for their role in causing gene silencing, a topic that has only emerged broadly across the scientific community in the past six years (Baulcombe 2004). These molecules usually are in the form of double-stranded (ds)RNA formed by sense and antisense transcripts or stem-loop structures within a single polymer.
 - 1.3.1.1 dsRNA gene-silencing by RNA interference (RNAi) (Denli and Hannon 2003) or RNAi-like pathways include siRNA and miRNA (22-26 nucleotides) to siRNA-directed DNA methylation (Baulcombe 2004) and DNA deletion (Mochizuki and Gorovsky 2004). Silencing instigated by dsRNA occurs in organisms of all biological kingdoms (Altuvia and Wagner 2000, Faugeron 2000, Tchurikov, et al. 2000, Hannon 2002).
 - 1.3.1.2 Once introduced into a model plant or animal, the effect of dsRNA is systemically spread throughout the organism and persists through the entire developmental period. Uniquely in humans, so far as is known from tissue culture experiments, siRNA species longer than 50 nucleotides cause a complete cessation of all gene expression (and thus effectively kill human cells) (Gura 2000). When worms ingest bacteria engineered to produce dsRNA, the corresponding gene is silenced in the worm (Timmons, et al. 2001). The dsRNA species is resistant to digestion in the worm's gut, destruction by enzymes in the bacterium, transmits to gut cells and then to deeper tissues and, eventually, to the germ line of the animal. The effects of a transient exposure to dsRNA in one generation can persist for at least two generations. In addition, siRNA from plants made transgenic for a marker gene in worms was capable of initiating gene silencing when injected into worms (Boutla, et al. 2002).
 - 1.3.1.3 While dsRNA has specific gene-targets, it is not always clear in advance what they will be (Zhao, et al. 2001, Jackson, et al. 2003, Semizarov, et al. 2003, Scacheri, et al. 2004, Jackson and Linsley). Thus it is critical to test the spectrum of genes that may be affected by even "specific" dsRNA molecules in the modified organism and any other organism that may be exposed to dsRNA from the modified organism by ingestion, grafting or pollination.
 - 1.3.1.4 The same dsRNA can have physiologically different effects at different concentrations. While very small quantities of dsRNA can induce silencing, dsRNA has nonspecific effects at lower concentrations than are required to induce silencing in, for example, zebrafish. These defects can be seen in embryos injected with as little as 30 picograms of dsRNA (Zhao, et al. 2001). This makes it imperative to assess the impact of the modification both on

expression of other genes in full knowledge of the amount of dsRNA that is being produced, and on all other relevant organisms at the full range of plausible concentrations to which they may be exposed.

- 1.3.1.5 Importantly, the *RNA effects are heritable even in multicellular animals, and transmit through food* (Cogoni and Macino 2000, Timmons, et al. 2001). Small RNA molecules developed in the food may have no effect on the plant itself, but could transmit to both somatic and germ cells in animal consumers. Therefore, microarray data is required to detect unintended and unanticipated effects on gene expression in both the modified maize and on consuming organisms.
- 1.3.2. The potential to inadvertently create novel RNA regulatory molecules, usually in the form of dsRNA, is too high by chance to ignore. They can be created by insertion of the transgene into a previously transcribed region (and not all transcripts emanate from ORFs), by aborted transcripts of the new transgene, read-through of terminator sequences, through fortuitous sequence similarity with an endogenous transcript, and by activation of a pseudogene. (For examples of such phenomena, see (Rang, et al. in press) and (Hirotsune, et al. 2003)).
 - 1.3.2.1 “[S]ilencing in response to a DNA transgene could still involve a dsRNA trigger: the transgene might integrate itself into the genome in such a way that a nearby promoter, or an inverted copy of the transgene itself, leads to the production of dsRNA, which could in turn enter directly into the RNAi pathway” (Mello and Conte Jr. 2004).
- 1.3.3. The Applicant has made prior claims that RNA “is generally recognized as safe (GRAS)”, and thus “the presence of...secondary RNA transcripts themselves raises no safety concern”⁶. But such claims are incongruous with the literature on small RNA molecules and gene silencing effects on duplicated genes initiated by mRNA (Meister and Tuschl 2004, Mello and Conte Jr. 2004). Surprisingly, the Applicant makes no mention of regulatory RNA in its 2002 assertion that RNA is GRAS, nor cites any research on RNAi or RNAi-like mechanisms of regulation.
 - 1.3.3.1 The claim that RNA is GRAS was made in defense of findings that event 40-3-2 in Roundup Ready Soybean had additional inserts beyond that initially reported to regulatory authorities before release.
 - 1.3.3.2 The presence of an additional 250 base pair fragment of the CP4 EPSPS gene went undetected in Roundup Ready soybean until 2001 (Windels, et al. 2001).
 - 1.3.3.3 In 2002, the Applicant argued that the only relevance of novel RNAs arising from read-through of the nos terminator of the full length insert into the newly discovered insert would be a novel protein and they could neither detect a novel protein using specific molecular tools nor detect the effect of a novel protein from their feeding studies.⁷ However, in an upcoming paper, Rang *et al.* report evidence “that these RNA variants [arising from read-

⁶p. 5 of MSL-17632, dated February 2002 (<http://www.food.gov.uk/multimedia/pdfs/RRSsafetysummary.pdf>)

⁷MSL-17632, dated February 2002 (<http://www.food.gov.uk/multimedia/pdfs/RRSsafetysummary.pdf>)

through of the nos terminator in Roundup Ready Soybean] might code for (as yet undescribed) CP4 EPSPS fusion proteins” (Rang, et al. in press).

- 1.3.3.4 Even more significant, Rang *et al.* found that “the read-through transcript was processed in four different RNA-variants” (Rang, et al. in press). The variants might arise from splicing pathways or other pathways. Post-transcriptional processing could signal entry into pathways that produce regulatory RNAs.
- 1.3.4. A significant concern raised by the authors about these variants, if they arise through a splicing mechanism, was that the nos sequence itself harbors a splice site. “The cis regulatory regions that initiate and mediate splicing are located within the removed region of spliced transcripts. If this is also true for the mechanisms mediating posttranscriptional processing of the described variants, it seems reasonable to assume that the transcribed nos terminator region might be responsible for processing the RNA. Since the nos terminator was and still is commonly used as regulatory region in the production of genetically modified crops, read-through products and RNA variants could also be expressed in these plants” (Rang, et al. in press). Since nos is also used in LY038, these new research findings require that it be evaluated for variant RNA species arising from the I-DNA.
- 1.3.5. Regardless of whether the variant RNAs arise from a cryptic splice site within nos or through other processing pathways, all novel RNA species in LY038 must be reported for a proper safety assessment.
- 1.3.6. Microarray descriptions should be capable of detecting novel RNA species in the modified plant, with the RNA source being the plant grown under a variety of relevant field conditions. The microarray should comprehensively represent the genomes of the cultivar of maize modified and unmodified. Since LY038 may be found in food, variant RNAs should be screened for effects on human tissue culture cells using a microarray for the human genome. The Applicant should then undertake both animal and human trials, but only if no effects are detected using tissue culture cells.
- 1.4 Creation of novel DNA sequences that may be harmful if taken into the human genome through food.
 - 1.4.1. If DNA molecules and proteins from GMOs persist in, and are taken up from the mammalian gastrointestinal tract (GIT), then this could, theoretically lead to development of chronic disease conditions. The fate and consequences of DNA persistence and uptake is, however, not extensively studied.
 - 1.4.2. It has generally been claimed that DNA and proteins are totally degraded in the mammalian GIT. This has been based on assumptions that had never been systematically examined (Palka-Santini, et al. 2003). A restricted number of recent publications have demonstrated that foreign DNA (and also proteins) may escape degradation at concentrations that allow persistence in the GIT and that such macromolecules may be taken up by the intestines, to circulate in the blood, and become seeded in internal organs (Schubbert, et al. 1994, Schubbert, et al. 1997,

Einspanier, et al. 2001, Hohlweg and Doerfler 2001, Klotz, et al. 2002). This has recently been demonstrated also in human volunteers (Forsman, et al. 2003).

- 1.4.3. Briefly summarized, the present conception of DNA persistence and uptake includes long fragments of ingested DNA. DNA may be detected in the faeces, intestinal wall, peripheral white blood cells, liver, spleen and kidney, and can cross placental boundaries to enter tissues of the fetus (Schubbert, et al. 1998), *and the foreign DNA may be found integrated into the recipient genome*. The state of GIT filling, and the feed composition may influence DNA persistence and uptake. Complexing of DNA with proteins or other macromolecules may protect against degradation.
- 1.4.4. Extrapolating from a number of experiments in mammalian cell cultures and in experimental animals, it is conceivable that in some instances insertion of foreign DNA may lead to alterations in the methylation and transcriptional patterns of the recipient genome, resulting in unpredictable patterns of gene expression and concentrations of gene products. Furthermore, even small inserts may result in a so-called ‘destabilization’ process, the end-point of which may be malignant cancer cells.
- 1.4.5. We therefore recommend that the Authority require a complete microarray description of the LY038 transcriptome compared to the unmodified control.
- 1.4.6. We also recommend that an analysis of the Glb1 promoter sequence for putative mammalian transcription factor binding motifs be supplied by the Applicant. We are concerned that upon *in vivo* uptake of DNA fragments by mammalian cells, and insertion into the recipient cell genome, the promoter may overexpress the cDHDPS gene, and/or deregulate the expression of endogenous genes. To what extent this hypothetical hazard may represent a real risk, and to which cell types the risk(s) is related, may be indicated by the repertoire of putative binding motifs in the promoter.

(2) Effects of lysine over-production

2.1 Maillard reaction and glycotoxins

- 2.1.1. Free lysine is a highly reactive amino acid which can undergo a variety of chemical reactions during food processing, notably the Maillard reaction (Meade, et al. in press). The Maillard reaction leads to a plethora of products upon food processing, including a wide variety of colour, flavour and aroma compounds, potentially beneficial compounds such as anti-oxidants, and toxic molecules (Fayle and Gerrard 2002).
- 2.1.2. Cooking and processing conditions can lead to the accumulation of toxic compounds from free amino acids. An example of such was demonstrated by two groups using asparagine, glutamine and methionine coupled with common reducing sugars to produce acrylamide from N-glycosides by the Maillard reaction (Mottram, et al. 2002, Stadler, et al. 2002). The ratio of safe to harmful Maillard reaction products is dependent on precise reaction conditions, including the

temperature, pH and concentration of starting materials. Thus changes to the concentration of lysine in the raw materials used to make food and feed may alter the toxicological properties of the resulting processed food.

- 2.1.3. An increasing body of work associates Maillard reaction products with inflammatory diseases of the gut. “An unrecognized risk factor for diabetic complications is the heat-generated group of advanced glycation end products (AGEs) which form in common foods during the spontaneous reactions between reducing sugars and proteins or lipids” (Vlassara, et al. 2002, p. 15596).
- 2.1.4. Thus an increase in lysine concentration may also lead to an increase in Maillard reaction products that are associated with inflammation, particularly within the growing community of diabetics (Vlassara, et al. 2002).
- 2.1.5. The Authority should request an analysis of Maillard reaction products or other glycotoxins that could arise from cooking or processing of LY038 corn. It is also essential that the Applicant supplement application A549 with a complete set of long-term, chronic, sub-chronic and acute toxicity feeding studies and allergenicity studies using cooked products derived from LY038.

2.2 Lysine and metabolism.

- 2.2.1. Recent evidence indicates that different proportions of lysine can have specific effects on metabolism.
 - 2.2.1.1 In a feeding study of young chickens, the authors observed a significant relationship between lysine, arginine and methionine. “These results suggest that more concern is prudent when considering the amino acid balance among [arginine, methionine and lysine] in the diet of young broiler chicks for maximum growth (and muscle creatine)” (Chamruspollert, et al. 2002, p. 660).
 - 2.2.1.2 In chickens, the effect is dramatic enough to result in a growth rate defect and a reduction in breast muscle creatine unless the diet is supplemented with arginine. While it has been known since the 1950s that elevated dietary levels of lysine affect arginine requirements in chickens, the connection to methionine has only recently become apparent.
 - 2.2.1.3 In a study on hypercholesterolemia⁸ in rabbits, it was found that higher concentrations of lysine and methionine, alone but particularly in combination, had a hypercholesterolemic effect (Giroux, et al. 1999). The effect was also correlated with changes in liver phospholipids. Higher supplements of arginine would sometimes mitigate the effect.
 - 2.2.1.4 In the rabbit study, it was possible to create conditions where the animals’ weights were not significantly changed under a dietary regime enriched for lysine and methionine but the symptoms of hypercholesterolemia were significantly different between this and a control group (Giroux, et al. 1999). Thus, feeding studies using high lysine corn must include such tests even on

⁸Note that this disease has also been associated with amyloidoses.

animals with no apparent differences in growth.

- 2.2.2. More research on the effects of dietary lysine in humans is required to evaluate potential effects of LY038 in the human food chain.
- 2.2.3. Animals feeding on plants have adapted to particular levels of total and free lysine and any derivative metabolites. In the case of humans, potentially toxic Maillard reaction products arising from cooking and processing can arise from a variety of amino acids, such as asparagine (Mottram, et al. 2002, Stadler, et al. 2002). Lysine is extremely reactive and can produce many toxic products (Fayle and Gerrard 2002). A change in the lysine concentration may generate new classes of toxins, or new concentrations of toxins that are presently extremely rare in our diets.
- 2.2.4. mDHDPS is feedback-inhibited by lysine, as are all known plant DHDPS enzymes (Azevedo and Lea 2001). Why this is the case is not explained by the Applicant, but there is evidence that higher levels of lysine alter the metabolism of the plants and could be harmful (Azevedo and Lea 2001). Lysine is a feedback inhibitor of aspartate kinase (AK), the first step in the common pathway leading to production of lysine off one branch, and isoleucine, threonine and methionine off the other (Azevedo and Lea 2001). While there is evidence of multiple isozymes of AK, the lysine-responsive isozyme is the major activity in maize (Azevedo 2002). Thus, plant biochemistry is responsive to lysine concentration. De-regulating DHDPS could cause fluxes in the amount of β -aspartyl phosphate available for the second branch and vary the concentration of these essential amino acids, proteins or other metabolites, or select for compensatory mutations.
- 2.2.5. Interestingly, the natural limitation to lysine accumulation in maize endosperm is lysine catabolism rather than limited production. The LOR-SDH lysine catabolic pathway first yields saccharopine by action of lysine 2-oxoglutarate reductase (LOR) and then amino adipic semialdehyde and glutamate via saccharopine dehydrogenase (SDH). Maize mutants, and mutants of *Phaseolus vulgaris* that accumulate lysine in seed, also have low LOR-SDH activity (Azevedo 2002).
- 2.2.6. “The amount of lysine that has been shown to be translocated to the developing endosperm for storage protein synthesis is 2 to 3-fold higher than what would be needed. Thus, accumulation of lysine in the soluble form would be expected, however, that is not the case, since the average concentration of lysine during endosperm development is kept low, *probably to avoid inhibition of [aspartyl kinase] activity and consequently methionine biosynthesis* [emphasis added]. These results suggested that soluble lysine concentration is mainly controlled by the rate of lysine catabolism instead of by the feedback inhibition of its synthesis” (Azevedo and Lea 2001, p. 267).
- 2.2.7. For whatever reason, cDHDPS has achieved a higher concentration of lysine in seed in line LY038 (MSL-19172), but this is not unprecedented and has been observed among other high lysine mutants of maize (Azevedo 2002).
- 2.2.8. The observation of lysine accumulation in seed is not consistent with a fully functional breakdown pathway. Since LY038 accumulates saccharopine but seems

to have normal levels of glutamate (MSL-19172), it may be that SDH catalysis of saccharopine hydrolysis is the rate-limiting step in the pathway or that SDH function has changed in LY038. This may be a compensatory change in LY038. Thus, the phenotype of any resulting hybrid cannot be predicted from the behaviour of LY038.

- 2.2.9. Normally this kind of effect could be considered environmental and not a food safety issue. However, this case is special. LY038 may be by luck or adaptation particularly resistant to the effects of high lysine. Hybrids that could form in nature may not be. The physiological sequelae of this metabolic stress could result in changes to the nutrient profile of hybrids that have implications for raw and/or cooked and processed products from these sources.
- 2.2.10. If the Authority were to recommend amending the Code to allow LY038, then it is also extremely important for the Authority to impose a condition in Column 2 of the Table to clause 2 of Standard 1.5.2 that *limits this approval to LY038 without extension to hybrid lines derived from LY038*. As in European regulation, all hybrids, whether between LY038 and an unmodified line or another approved modified line, must in this case be treated as a new organism requiring a full safety evaluation.

(3) Limitations in knowledge will require post-launch monitoring.

- 3.1 In this Part of our submission, we have highlighted several serious limitations in knowledge about the effects of the LY038 event on food. These limitations will not be overcome quickly through more molecular descriptions of the insertion, the composition of LY038, or bioinformatics. We will therefore argue for additional testing and, if applicable, post-launch monitoring.
- 3.2 Allergenicity is arguably the most complex and difficult assessment to make (Pusztai, et al. 2003). That is why we believe that if LY038 passes more extensive animal tests, then human trials are essential as part of an application package to FSANZ. To be valid, they must be conducted in controlled settings, not on consumers.
- 3.3 The Applicant should conduct allergenicity studies using a wide range of mildly and highly processed and unprocessed foods made from the GMO itself.
- 3.4 Allergenicity studies must not be limited to dietary exposure for foods that are commonly sold as powders or which may become airborne during routine processing by the consumer. The study should include an assessment of inhalation challenge as well as dietary challenge.
- 3.5 Until now, a *de facto* passive surveillance of novel foods has substituted for an active monitoring of genetically engineered foods. For example, it is common to hear the claim that “Americans have been eating GM for years and they seem alright.” However, passive surveillance of consumers falls short of what we would recommend for New Zealand, because passive surveillance:

- 3.5.1. detects only “serious acute adverse effects” and is not appropriate for the kinds of effects most likely to arise from novel foods (Schilter and Constable 2002);
 - 3.5.2. lacks an intrinsic control population from which baseline data of chronic conditions can be extrapolated;
 - 3.5.3. could incur delays if chronic conditions were misdiagnosed;
 - 3.5.4. is not designed as a failsafe for unanticipated errors, such as, for example, the adventitious contamination of novel foods with novel feeds approved only for animals.
- 3.6 We recommend that a risk management plan should be submitted by the Applicant as part of A549. That plan should include a description of how the Applicant will conduct post-launch monitoring (Schilter and Constable 2002). Failure to do so would, in our minds, undermine the Authority’s objective of ensuring that consumers receive “adequate information relating to food” so that they may make informed choices in the long-term.

Part Two: Data assessment

(4) Molecular characterization of Cre-recombinase line.

- 4.1 We argue that assessing the proposed amendment to Standard 1.5.2 requires a separate and formal evaluation of the Cre-recombinase progenitor line that includes LY038, as well as the provision of the proper molecular data for this progenitor line in an evaluation of LY038.
- 4.2 LY038 is a hybrid⁹ line of corn derived from a cross between pre-LY038, the *cordapA*-containing plants, and an undescribed transgenic corn line only referred to as the Cre-recombinase donor. That donor is a transgenic maize plant modified by the insertion of at least the *cre* recombinase and *nptII* transgenes (and possibly other undisclosed insertions).
- 4.3 We will make the case that molecular characterization of LY038 with respect to the nature of the modifications potentially introduced from the Cre-recombinase line is incomplete in certain important aspects. These should be made complete before a decision is reached on whether the Code should be amended.
 - 4.3.1. According to Standard 1.5.2 – Food Produced using Gene Technology, “A food produced using gene technology, other than a substance regulated as a food additive or processing aid, must not be sold or used as an ingredient or component of any food unless it is listed in Column 1 of the Table to this clause and complies with the conditions, if any, specified in Column 2.” To our knowledge, the Cre-recombinase donor is not listed in the Table to Clause 2. Therefore, the Authority must require a complete analysis of the Cre-recombinase line or be satisfied that the analyses submitted in support of LY038 make such an analysis redundant.
 - 4.3.2. We are presently not convinced that the analyses currently submitted are sufficient to constitute a retrospective analysis of the Cre-recombinase line.
 - 4.3.3. Up to twenty chromosomes (the 2N number for maize) may have been changed in the creation of the Cre-recombinase line, due to unanticipated and uncontrolled insertions of partial fragments of plasmid PV-ZM003. Ten of these chromosomes would have been present in the F1 hybrid between pre-LY038 and the Cre-recombinase donor. Assuming that the full length *cre* and *nptII* genes from PV-ZM003 were linked on a single chromosome, then by the F3 generation (from which LY038 derives), up to 11 chromosomes would be from pre-LY038 and up to 9 from the Cre-recombinase line. Of course extensive crossing over during meiosis makes even this a simplistic analysis. It is possible for DNA to have crossed over between all the Cre-recombinase line and pre-LY038 chromosomes.
 - 4.3.4. Probes 14 (18), 15, 16 (20), 17, 19 and 21-23 (Figures 17-18 of Monsanto Australia Limited 2004) were used to generate evidence that LY038 was devoid of DNA

⁹Figure 5 of Monsanto Australia Limited (2004). Application to Food Standards Australia New Zealand for the inclusion of Lysine maize LY038 in Standard 1.5.2 – Food Derived from Gene Technology.

sequences that might derive from PV-ZM003. We note the following problems with this analysis that should be clarified by the Applicant.

- 4.3.4.1 The size of the probes ranges from 0.3 to 1.9kb, with most being 1.2 to 1.9 kb. The Applicant should report the minimum size of target DNA that all probes will detect at a minimum stringency of 0.5 copies per genome (see Figure 1). Partial fragments of I-DNA or genomic DNA interspersed into I-DNA have been detected in fragments as small as 15 bp (Svitashev, et al. 2002).
- 4.3.4.2 Even inserts of this small size can be biologically relevant. 15 nucleotides is 5 new codons, or could create a new transcription factor binding site. Any one of the new codons could be a stop codon. Multiples of nucleotides other than 3 will put shift the reading frame. Toxic proteins can be produced by truncated mRNAs (Wilusz, et al. 2001), themselves often the product of insertions¹⁰.
- 4.3.4.3 The Applicant need meet the dual standard of demonstrating comprehensive coverage in their search for PV-ZM003 DNA (which the list of probes does) and demonstrating appropriate sensitivity to small inserts (which the data does not).
- 4.3.4.4 PCR data does not substitute for Southern data because small fragments cannot be expected to insert in the correct order or proximity for easy amplification.
- 4.3.4.5 Importantly, there are a number of light ('ghost') bands clearly visible in lanes marked LY038(-) in Figure 19 (see Figure 2). This blot is a simultaneous hybridization with all probes 14-17, spanning rACT1, cre, Zm.hsp70 intron and NOS3'. Since LY038(-) is a negative segregant of functional *cre* and *nptII* genes, these ghosts could be indicating the presence of partial plasmid sequences distributed throughout the genome of the LY038 line. At a minimum, the Applicant should have included the parental controls for comparison. Preferably, the Applicant would have cloned and sequenced the ghost bands to determine if they had uncharacterized insertions.
- 4.3.4.6 We are confused by the Applicant's conclusion (Monsanto Australia Limited 2004, p. 20) that "Hybridisation of Lysine maize LY038 DNA digested with Spe I (Lanes 3 and 9) produced an expected unique band of approximately 3.8 kb in addition to those produced by the LY038(-) control DNA (Lanes 1 and 5)". Our confusion stems from the apparent band in this 3.8-4.1 region in LY038(-) lanes. The co-migrating band in LY038(-) should be sequenced before the Applicant can conclude that "[t]his band resulted from the hybridisation of the rAct1 intron portion of the probe with the rAct1 intron associated with the cordapA cassette", because it could be that and a second, *uncharacterized*, insertion in the homologous chromosome.
- 4.3.4.7 Contrary to the Applicant's conclusion (Monsanto Australia Limited 2004, p. 20) that "Hybridisation of Xho I and Xba I-digested Lysine maize LY038 DNA (Lanes 4 and 10) produced a single unique band of 3.5 kb in addition to

¹⁰This is one reason that we argue, below, that a complete description of the transcriptome should be provided by the Applicant.

the background observed in LY038(-) (Lanes 2 and 6)”, we count 2 bands unique to lane 10 (LY038). The larger band, somewhere between 15 and 20 kb, may be a marker of a chromosome absent from LY038(-) and of Cre-recombinase line origin.

- 4.3.4.8 We would like confirmation that a ghost band visible in Lane 4 (DNA from LY038) at approximately 3.1 kb is an artefact to support the Applicant’s assertion (Monsanto Australia Limited 2004, p. 21) that “Hybridisation of Lysine maize LY038 DNA digested with...the combination of Xho I and Xba I (Figure 21, Lanes 4 and 10) showed no detectable band.” We reproduce Figure 21 (see Figure 3) to highlight the band in question. The observation was not reproducible because the band should also be visible in Lane 10, but is not. However, no positive control for hybridization is included in Lane 10, making it difficult to dismiss the importance of the band in Lane 4.
- 4.3.5. The sensitivity of analysis of genomes for insertions of partial transgenes must be at least to the standard of published studies that have been able to demonstrate their much higher effectiveness at detecting unexpected inserts (Svitashev, et al. 2002, Makarevitch, et al. 2003). A combination of FISH, fiber-FISH and Southern analysis was used in these studies, whereas the Applicant has only used Southern and PCR. These other studies found that even to their much higher standard, they failed to detect all insertions initially. They found that “[t]ransgenic oat line 3830...was previously characterized with FISH, fiber-FISH, and Southern analyses and shown to have a single major transgene locus estimated to be ca. 15 kb in length. However, when T1 progeny of line 3830 were analyzed by Southern blot hybridizations with longer exposure times and more genomic DNA per lane compared to these previous analyses, two additional minor transgene loci were detected” (Makarevitch, et al. 2003). Their work emphasizes how vulnerable analyses are to arbitrary exposure times, probe sizes and wash stringency.
- 4.3.6. In addition to detecting multiple insertions, these authors sequenced around insertion sites and found evidence of transformation-induced genomic rearrangements and deletions that again would not be detected by Southern blotting using transgene-specific probes.
- 4.3.7. In their commentary on detecting the many small and/or complex products of multiple insertion sites, Kohli et al. (2003) said: “Mehlo *et al.* (2000) studied seven transgenic maize lines with multicopy transgene loci and found that every line showed some form of transgene rearrangement in at least one copy. Importantly, some of these rearrangements could be detected by sequencing and/or PCR, but were too subtle to be picked up by Southern blot analysis, the predominant technique used to characterize transgene loci. The authors speculated that undetected ‘minor’ rearrangements might be extremely common...However, sequencing and PCR analyses by themselves would provide an incomplete picture of transgene organization because, depending on the location of the sequencing and PCR primers, some major rearrangements might not be detected. Therefore, *PCR, sequencing and hybridization provide complementary information regarding locus structure* [emphasis added]” (Kohli, et al. 2003).

- 4.3.8. Additional evidence is also required to verify the Applicant's claim that "[t]he excised *nptII* gene cassette (circular extragenomic DNA), which did not contain an origin of replication, was subsequently lost, most likely through cell division" (Monsanto Australia Limited 2004, p. 7). It is not always true that the DNA between *loxP* sites is lost. In a similar strategy to A549, transgenes in wheat were removed by crosses with a Cre-recombinase donor (Srivastava and Ow 2003). The excised *and retained* DNA was only detected using PCR, not Southern blotting. While these transgenes did not have recognized replication sequences, there is the possibility that excised circles replicated (Srivastava and Ow 2003). Depending on the efficiency of replication, *nptII* DNA might persist at less than an average of 1 copy per genome across the cells of the transgenic plant. The authors only pressed their Southern analysis to approximately 0.5 copies per genome rather than, say, 0.01 copies per genome (or one gene per 100 cells).
- 4.3.9. Moreover, excision does not necessarily eliminate all effects of the original insertion. Reliable combinations of *cis*-acting sites and excision enzymes can still produce excision products that differ from both normal and expectation. Exceptions are often such because it is harder to detect events that have been processed differently. In one such case, the excision of adenovirus ad12 from hamster tissue culture cells, very minor variations of the excision event had spectacularly different biological consequences (Pfeffer, et al. 1999). In that case, the oncogenic phenotype conferred by the insertion of the virus was retained after excision of the virus. Even the subclones that carried "only minute fragments from the right terminus of Ad12 DNA, retain[ed] the oncogenic phenotype. Each subclone of the hamster tumor cell lines seems to exhibit a different pattern of persisting tiny Ad12 DNA segments" (Pfeffer, et al. 1999, p. 114). Extreme care must be exercised by the Authority in evaluating the DNA sequence surrounding all insertion sites including those processed by the cre recombinase.
- 4.3.10. Although we find evidence of LY038 I-DNA locus amplification by PCR, e.g. Figure 22, we cannot find an instance in this application where LY038 DNA has been interrogated using PCR and primers for *nptII* or *cre*, despite repeated statements in the application that loss of *nptII* and *cre* was confirmed using both Southern and PCR techniques¹¹. We believe that it is essential that the Applicant make the PCR data for the cre-recombinase-specific markers clear.

(5) Molecular characterization of LY038.

- 5.1 We will make the case that molecular characterization of LY038 with respect to the nature of the I-DNA in the "*cordapA*-containing plants" and *in planta*-produced cDHDPS is incomplete in certain important aspects. These should be made complete before the decision is made whether to amend the Code.
- 5.2 Probes 1-13 (Figures 2-3. Monsanto Australia Limited 2004) were used to generate

¹¹"Through extensive PCR screening of subsequent maize breeding progeny, the *cre* gene was segregated away from the *cordapA* gene such that F3 progeny containing only the *cordapA* gene cassette in the inserted DNA (I-DNA) and lacking both the *nptII* and the *cre* gene cassettes were identified and designated as Lysine maize LY038" (p. 7).

evidence that the LY038 genome has only one complete copy of I-DNA derived from PV-ZMPQ76. This analysis should be supplemented by the Applicant in the following ways.

- 5.2.1. The size of the probes ranges from 1.3 to 1.6 kb. The Applicant should report the minimum size of target DNA that these probes will detect at the stringency of 0.5 copies per genome (see Figure 1). Partial fragments of I-DNA or genomic DNA interspersed into I-DNA have been detected to fragments as small as 15 bp (Svitashev, et al. 2002).
- 5.2.2. As indicated above, PCR data does not substitute for Southern data because small fragments cannot be expected to insert in the correct order or proximity for easy amplification.
- 5.2.3. Importantly, there are a number of ghost bands clearly visible in lanes loaded with LY038(-) DNA using probe 5, spanning the Glb promoter (Figure 9, Monsanto Australia Limited 2004). Since LY038(-) is a negative segregant, presumably missing the *cordapA* gene, these ghosts could be indicating the presence of partial plasmid sequences distributed throughout the genome of the LY038 line. At a minimum, the Applicant should have included the parental controls for comparison. Preferably, the Applicant would have cloned and sequenced the ghost bands to determine if they had uncharacterized insertions.
- 5.2.4. We are confused by the Applicant's conclusion (Monsanto Australia Limited 2004, p. 18) that hybridisation of LY038 DNA using probe 5 produces a unique band in addition to the background observed in LY038 (-) (Figure 9, Monsanto Australia Limited 2004). This statement implies that all but one band should be in common between the genomes of LY038(-) and LY038, but there is clearly a band of 1.5 kb that appears in the LY038(-) control DNA and that is not observed in LY038 genomic DNA. In MSL-19109 the Applicant concludes that "this hybridization could be a result of variances in the genetic background" (p. 22 of 69). At a minimum, the Applicant should have included the parental controls for comparison, and sequenced the DNA of this band to confirm its identity.
- 5.2.5. The Applicant should meet the dual standard of demonstrating comprehensive coverage in their search for PV-ZMPQ76 DNA (which the list of probes does) and demonstrating appropriate sensitivity to small inserts (which the data does not). Further characterizations of the LY038 genome are required to the standard we discussed in section 4, above.
- 5.2.6. Finally, the Authority should be aware that processing of *loxP* sites does not entirely reverse the effects of the original insertion nor leave the site with the same risk spectrum as before the insertion of *loxP* sequences. Processing leaves an intact *loxP* sequence in the chromosome. This sequence may make the chromosome vulnerable to double strand breaks should, by chance, LY038 produce hybrids with a *cre* containing line [or the *cre* recombinase gene or activity ever again transfer to LY038 (e.g. by horizontal gene transfer)]. This is one reason that we argue, should the Authority recommend Option 2, a condition be placed in Column 2 of the Table

to Clause 2 restricting approval to LY038 and not to any derivative hybrids.

- 5.3 MSL-18365 and MSL-18585 attempt to demonstrate equivalence between maize DHDPS (mDHDPS), *E. coli*-produced cDHDPS and *in planta*-produced cDHDPS.
- 5.3.1. In MSL-18365 the authors conclude that “[t]he introduction of cDHDPS protein is functionally similar to the maize DHDPS enzyme” (p. 11 of 49). Beyond citing a 1991 study by Frisch *et al.*¹² which discusses mDHDPS, neither this study nor MSL-18585 can draw any conclusions about comparisons between mDHDPS and any other DHDPS because mDHDPS was never included in the analyses presented. Before such statements are accepted by the Authority, the Applicant should submit supporting data.
- 5.3.2. The phrase “functionally similar” is vague. If functional similarity is restricted to structural characteristics, then, as we noted in Part One, the eukaryotic and prokaryotic DHDPS have different quaternary structures, and this can have important implications for safety assessment. We also speculate that the potential to form amyloids may be greater for *in planta* cDHDPS than either *E. coli*- or *C. glutamicum*-produced cDHDPS because of the expected pH fluctuations in the chloroplast.
- 5.3.3. If functional similarity is restricted to biochemical parameters, such as specific activity, K_m , or V_{max} , then these should be reported for all *in planta*-produced DHDPS proteins. Only specific activities of *E. coli*- and *in planta*-produced cDHDPS were reported and these, unfortunately, do not establish similarity.
- 5.3.3.1 In MSL-18585 “[t]he specific activity for the plant-produced and *E. coli*-produced cDHDPS protein was estimated to be 68 ± 3 and 84 ± 5 U/mg total protein, respectively.” Importantly, the authors concluded in MSL-18365 that the specific activity of the enzyme from MSL-18365 was 107 ± 6 U/mg of protein, or approximately 23 to 40 U/mg different from the *E. coli*- and *in planta*-produced cDHDPS, respectively. In published studies, differences in specific activity of comparable magnitudes to those in MSL-18365 and 18585 have been considered significant (e.g. Bessler, et al. 2003).
- 5.3.3.2 Simple statistical analyses will demonstrate that, based on a replication power of four per assay, the reported specific activities are significantly different¹³.
- 5.3.3.3 Measures of specific activity can suffer from a high variance. With this in mind, we suggest that the specific activity data is inappropriate for drawing conclusions of identity or functional similarity, other than to say that the isolated proteins perform the same conversion of substrate into product in this *in vitro* assay. Either the specific activity measures are significantly different and refute the suggestion of functional similarity, except by the most liberal of definitions of similarity, or they are unreliably variable and better measures, such as K_m and V_{max} , should be provided.

¹²Note that the bibliography incorrectly lists the first author as Frish, D.A.

¹³Student T test $p=0.003$.

- 5.3.4. Functional similarity could instead be interpreted to mean that the reaction mediated by cDHDPS is functionally similar to that mediated by mDHDPS. However, cDHDPS is relatively insensitive to lysine feedback inhibition, the central attribute of this enzyme for purposes of maize transformation. In our view, it is a matter of judgment how to prioritize biochemical functions as more or less significant. Contrary to the Applicant, we would consider the difference in lysine feedback inhibition and its metabolic consequences as significant and not similar to the properties of maize DHDPS.
- 5.3.5. The reaction an enzyme performs is influence by the environment. Observing a common reaction product between two enzymes in the same environment is not a positive identification of the range of products the enzyme will make in different environments. “[T]here is an emerging body of evidence suggesting that an enzyme’s functional characteristics can also be affected by its metabolic context and that temporal and spatial dynamics of enzyme interactions can be important determinants of enzyme functionality” (Heilmann, et al. 2004).
- 5.3.6. In study MSL-18365, the authors report two co-purified proteins of different molecular weight (32 and 34 kDa, respectively) isolated from *E. coli* transformed by an expression plasmid encoding a proxy of the *in planta* construct.
- 5.3.6.1 The 32 kDa protein is likely to have the same amino acid sequence as native cDHDPS as indicated by a combination of techniques. By themselves, none of the analyses conducted provided a complete characterization of the entire protein, though some, such as peptide sequencing, could have. We see no reason not to provide a complete view of this protein beyond that it might be inconvenient or expensive for the Applicant.
- 5.3.6.2 Proteins with common start sites but of different size can have different biological properties. For example, the gene for GP protein in Ebola virus produces two variant GP proteins with common amino termini. “GP is a membrane glycoprotein that is located at the surface of EBOV-infected cells and forms the spikes on virions. Expression of GP, which is encoded by two overlapping reading frames, requires the insertion of a non-template-coded adenosine residue by a mechanism of transcriptional RNA editing. Most (about 80%) GP mRNAs are not edited, and they direct synthesis of the nonstructural glycoprotein sGP, which is secreted from EBOV infected cells. GP and sGP are identical at their NH₂-terminal ends (295 amino acids) but differ at the COOH termini owing to the use of different reading frames” (Volchkov, et al. 2001).
- 5.3.6.3 In the Ebola example, RNA editing is part of the mechanism that makes for two polypeptides from the same sequence of DNA. Alternative splicing can also create these effects. More troubling, the Ebola mechanism could not be determined in advance from an analysis of the GP gene. Only transcriptome and proteomic data will detect variant species of RNA or polypeptides.
- 5.3.6.4 In section 7.1 the authors report detecting additional immunoreactive proteins of less molecular weight than 32 kDa. They allude without evidence or citation to the identity of these proteins as “degradation products of the

- cDHDPS protein” (p. 20 of 49). Moreover, in this description it is claimed that the identity of the smaller proteins was confirmed during “development work”.
- 5.3.6.5 The Authority should insist that the identity of these proteins as degradation products be confirmed and done so formally in a set of experiments that include all relevant controls.
- 5.3.6.6 The identity of the 34 kDa protein is never made definitively, but the authors suggest that it is a contamination product of the 32 kDa protein (but the basis for this assertion is not clear).
- 5.3.6.7 We are troubled by the Applicant’s failure to detect the 34 kDa protein using the goat anti-cDHDPS antiserum. This point will be raised again in consideration of MSL-18585.
- 5.3.6.8 We are also concerned that we cannot find any description of the origin of this antiserum in the materials provided to us.
- 5.3.6.9 The Applicant must report whether the antiserum was raised against *E. coli*-produced cDHDPS, *C. glutamicum* DHDPS, or *in planta*-produced cDHDPS.
- 5.3.6.10 Was the antiserum affinity purified? If so, the Applicant may have lost any antibodies that would bind to antigens unique to *in planta*-produced cDHDPS.
- 5.3.6.11 How many exposures and how frequently were goats exposed to the antigen(s)? What antibody classes are in the serum? Could other classes of antibodies mask epitopes from those classes used in the detection assay?
- 5.3.6.12 The origin of the antiserum is critical information needed to evaluate the claim that the gene product in plants is entirely of the structure and function expected.
- 5.3.6.13 The complete characterization of the 34 kDa protein should be possible and required by the Authority, because its existence makes ambiguous the Applicant’s claims about the nature of the active form of the enzyme and its physicochemical properties.
- 5.3.7. Even equating primary structures is an imperfect gauge of biochemical equivalence. A proper understanding of enzyme behaviour requires isolating it from the relevant cellular location.
- 5.3.7.1 As introduced above, “there is an emerging body of evidence suggesting that an enzyme’s functional characteristics can also be affected by its metabolic context and that temporal and spatial dynamics of enzyme interactions can be important determinants of enzyme functionality” (Heilmann, et al. 2004). The physical similarity between cDHDPS and *E. coli*-produced cDHDPS, or between the latter and *in planta* cDHDPS will not capture the potential effects of regiospecificity on functionality and substrate specificity that are unique to the environment of the plant cell in general, or the chloroplast in particular.
- 5.3.7.2 Indeed, when an *Arabidopsis* 16:0 Δ^7 desaturase protein was maintained in the cytoplasm of the cell rather than the chloroplast, it still produced its normal product but *changed the diversity of products* it produced (Heilmann, et al. 2004). The same effect was achieved by targeting cytoplasmic desaturases to

the chloroplast. *No structural analysis alone will predict the effect of context on an enzyme or its potential to produce unanticipated products in a novel context. Therefore, structural analyses equating E. coli- and in planta-produced cDHDPS cannot substitute for the use of in planta-produced cDHDPS in all biochemical and feeding experiments.*

- 5.3.8. The identity of *in planta*-produced forms of cDHDPS is critical and the Applicant must demonstrate attention to all factors affecting this determination. The three principle techniques used by the Applicant were peptide sequencing, MALDI-TOF and immunoreactivity. Anomalies or deficiencies in procedure are noted below for all these approaches.
- 5.3.9. Results of N-terminal sequencing. The Applicant determined the first few amino acids from proteins of apparent molecular weight 33 kDa. From this size class, two distinct N-terminal sequences were detected. This variety of peptide forms is considered by the Applicant to be unremarkable because it has been seen before.
- 5.3.9.1 However, the Applicant cites evidence from three Monsanto internal reports rather than the peer-reviewed literature.
- 5.3.9.2 The evidence in these reports is not appended to this application and so could not be evaluated.
- 5.3.9.3 Finally, that three other transgenes produced a variety of N-terminal peptide types is not evidence that the variety is normal and safe.
- 5.3.10. What would be more reassuring is an explanation of how these types arise and an approach to peptide purification that could detect other variants that arise from post-transcriptional or post-translational processing.
- 5.3.11. We consider that the Applicant could have discovered this reported variety of polypeptides only by sequencing and therefore may have missed other species of this protein. Coupled with the unresolved identity of additional immunoreactive polypeptides (discussed in the following section), this suggests that the Applicant may not have made positive and comprehensive identifications of all novel proteins arising from the modifications of LY038.
- 5.3.12. Confirmation of identity using goat anti-cDHDPS antiserum (Lot 7104510). We reiterate from above our concern that the Applicant has not, to our knowledge, disclosed the methods used to produce this antiserum. As we discussed in Part One, without a full 2D proteomic survey of novel proteins in LY038, we cannot begin to exclude the very real possibility that a variety of variant forms of cDHDPS, with variation introduced through alternative splicing and post-translational modifications, have been missed by the Applicant. Some of these forms may be invisible to antisera raised against protein sourced from bacteria (see our discussion in the next section for references).
- 5.3.12.1 The authors report (MSL-18585, p. 21 of 46) that a “few additional immunoreactive bands of lower molecular weight were observed...for both the plant-produced and *E. coli*-produced cDHDPS proteins” and assert that “[t]hese bands were identified previously as degradation products of the

- cDHDPS protein”, citing study MSL-18365. This conclusion is not justified by the findings in MSL-18365.
- 5.3.12.2 As we indicated above, the Applicant provided no evidence in MSL-18365 that the smaller proteins were indeed degradation products. In MSL-18365, the authors state that “a few additional immunoreactive bands of lower molecular weight were observed...these bands were identified during development work as degradation products of the cDHDPS protein” (p. 20 of 49). The authors of MSL-18365 referred to conclusions drawn during development work that, to our knowledge, have not been published or provided for evaluation by anyone.
- 5.3.12.3 MSL-18365 was a study that included no material from plants and therefore provides no evidence to support the conclusion that *in planta*-produced lower molecular weight bands are degradation products of full size cDHDPS.
- 5.3.13. There remains the possibility that the Applicant failed to detect all potentially immunoreactive forms of protein. Glycosylation (discussed in detail in the next section) or its absence can create different antigenic epitopes from those present in otherwise identical polypeptides. For example “O-linked glycosylation has a profound effect on the antigenic properties of peptides. O-linked glycosylation can generate a neo-epitope (e.g., CII), or can have as an effect the hiding of an epitope (e.g., VF13N). O-linked glycosylation can mimic other epitopes (molecular mimicry of cytokeratins). It can change the properties of an epitope even without really being part of the epitope (CD43 and GPA)” (Van den Steen, et al. 1998).
- 5.3.14. The identity of all novel proteins produced *in planta* directly or indirectly because of the modification or choice of recipient is the absolute obligation of the Applicant. The Authority should not relax its standards on these identifications, particularly as making proper identifications on these alleged degradation products is squarely within the bounds of biochemical techniques available to the Applicant.
- 5.3.15. The authors attempted to detect glycoforms of *in planta*-produced cDHDPS. Glycoforms of a protein are sugar-modified variants of the same primary amino acid polymer. We argue that all variants, not just glycoforms, must be characterized because different forms can have different biochemical characteristics. MSL-18585 has failed to convince us that all variants of cDHDPS arising from post-translational modifications of *in planta*-produced cDHDPS have been detected. The mass spec data reassures us that the purified protein has no glycoforms, but again, the Applicant should identify all novel proteins produced in LY038, not just present the protein that is concentrated by their purification scheme.
- 5.3.15.1 Post-translational modifications are alterations to the chemical structure of a polypeptide that are not controlled by the reactions in translation. They are therefore not specified in the DNA sequence for a gene. More than 300 different types of chemical modifications are known, and they are distributed among the following: phosphorylation, glycosylation, acetylation. No modification is exclusive so combinations of modifications and groups of modifications can co-exist on the same protein (Norregaard Jensen 2004).
- 5.3.15.2 Glycosylation. The three main post-translational protein modifications that

use carbohydrates are N- and O-linked glycosylation and glycosyl phosphatidyl inositol (GPI) anchors (Van den Steen, et al. 1998). Over half of all proteins are glycosylated (Van den Steen, et al. 1998). A single protein can emerge with a large variety of different glycoforms despite being synthesized in the same cell at the same time (Rudd and Dwek 1997). There may be many more forms of glycosylation than discussed above (Manzi, et al. 2000). Little is known about these other forms of modification, but procedures have been developed to isolate proteins with such modifications (Manzi, et al. 2000).

- 5.3.15.3 *Glycosylation is a significant complexity in protein analysis.* It is critical that the Applicant fully characterize glycosylation for two reasons. First, failing to do so can invalidate subsequent analyses that use procedures or reagents dependent on knowing the full glycosylation status of the *in planta*-derived protein. Second, the biochemical characteristics of proteins with different types or amounts of glycosylation are factors of safety.
- 5.3.15.4 Confidence in chemical purification of all glycoforms. Glycosylation can change the characteristics of a protein in such a way as to cause it not to be detected by methods that rely on the overall chemical properties of the protein or on antibodies. Various glycosylation patterns can lead to different biochemical and antigenic properties. It would be much better if the Applicant had first developed a glycoprofile of LY038 (Manzi, et al. 2000), and worked from that to an identification of all cDHDPS variants.
- 5.3.15.5 Glycosylation can significantly change the molecular weight and charge profiles of proteins; both of these parameters are relevant to the Applicant's isolation method.
- 5.3.15.6 For example, in a study comparing glycoforms of the CJD prion-forming protein it was found "that human brain and CSF [species of] PrP^C exist [as] full-length and truncated species that exhibit variable degrees of glycosylation, giving rise to over 60 charge isomers" (Castagna, et al. 2002, p. 344).
- 5.3.15.7 Glycoforms of the Prp^C protein in humans, for example, can alter the apparent molecular weight by 50% (Castagna, et al. 2002). If the minimum molecular weight of *in planta*-produced cDHDPS were 33 kDa, then possible variants could weigh in as high as 50 kDa.
- 5.3.15.8 It is not made adequately clear from the METHODS how glycosylation might affect the Applicant's isolation procedure and whether all glycosylated forms of the protein would be fairly represented in the final stock solution.
- 5.3.15.9 Purification based on activity would specifically fail to detect glycoforms with low or no activity in the test assay, but which still present a risk as allergens or novel activities.
- 5.3.15.10 Therefore, the cDHDPS purification methods used by the Applicant should be quality assured to concentrate all glycoforms. Putative glycoforms could be identified in a full proteome comparison of LY038 and an unmodified control. Failing this, their result demonstrating an absence of glycosylation could be a false negative result due to exclusion of one or more minor forms that are glycosylated.

- 5.3.15.11 We note that in application A524 (Food derived from herbicide-tolerant wheat MON 71800, subsequently withdrawn by the Applicant) the Applicant augmented their isolation procedure with a second, immunological isolation and purification procedure. Although neither of these approaches is perfect, both should be used.
- 5.3.16. Factors of safety. Different glycoforms can have different biochemical characteristics. For example, different strains of prions (e.g. the causative agent of Mad Cow Disease and vCJD) derive from different glycosylation patterns (discussed in Rudd, et al. 1999).
- 5.3.17. We recommend that the Applicant prepare or provide a complete proteomic analysis of LY038 accounting for all changes. Each change should be identified as either a variant of cDHDPS or an unintended change in the modified plant. All variant forms of cDHDPS should be characterized for glycosylation or other post-translational modifications.
- 5.3.18. Alternative splicing can create other variants. “The diversity of gene products originating from a single gene is mainly due to alternative splicing of transcripts and co- and post-translational modification of proteins. The human genome is predicted to contain on the order of 30,000 open reading frames, each of which, on average, may produce five or six different mRNA species. Each of these mRNA species is in turn translated into proteins that are processed in various ways, generating on the order of 8–10 different modified forms of each polypeptide chain. Thus, the human genome may potentially produce on the order of $(30\,000 \times 6 \times 10)$ 1.8 million different protein species” (Norregaard Jensen 2004). To our knowledge, this observation is not unique to humans and may well include maize.
- 5.3.19. Errors are one source of alternative transcripts. Exon skipping occurs if spliceosome components formed at the 5’ end of one exon interact with the 3’ components of a non-adjacent exon. Given the weak consensus around important splice sites, it is possible that upon occasion some sequences are mistaken as splice sites.
- 5.3.20. Not all alternative splice products are thought to be errors. Some transcripts always give rise to different mRNAs. Alternative splicing may also be regulated, arising only in certain kinds of cells or under certain physiological conditions. Examples:
- 5.3.20.1 A single gene in the fly *Drosophila*, DSCAM (Down syndrome cell adhesion molecule), produces some 38,000 different mRNAs from alternative splicing (Schmucker, et al. 2000). “The isolation of multiple cDNAs from rat and cow revealed that over 1000 different neurexin mRNAs could potentially be synthesized from three genes by virtue of alternative promoter usage and alternative splicing” (Graveley 2001).
- 5.3.20.2 Alternative splicing may do more than just mix and match exons in different transcripts. The exon may be skipped or include intron sequences (exon extended), or the transcript may include an intron as an exon (Cartregni, et al. 2002).
- 5.3.21. Alternative transcripts and start codons can arise unexpectedly from transgenes

and, in some cases do not support enough protein product to be detected by protein-level analysis (Burke, et al. 2000). There is new evidence suggesting that the nos terminator sequence used in LY038 and most commercial GMOs is a splice site (Rang, et al. in press) and a recombination hotspot (Kohli, et al. 1999).

5.3.22. The proteomic data recommended above should be supplemented with microarray data to complete the description of the LY038 and parental transcriptomes.

5.3.23. Description of the transcriptome would also increase the chances of detecting any unanticipated changes in gene expression at other loci. *De novo* methylation often occurs in GMOs, and methylation is one mechanism of gene silencing. Methylation changes have been directly attributed to transformation of plants with transgenes (Weld, et al. 2001) and later through creation of hybrids with GMOs.

5.3.23.1 “Using two stable rice lines with introgression from wild rice, *Zizania latifolia*, we found marked changes in DNA methylation and transcription in several cellular genes and transposon (TE)-related segments had occurred compared with the rice parent. Changes in methylation and transcription was also observed in an independently produced asymmetric somatic hybrid of rice and *Z. latifolia*, which involves a different rice genotype, but also contains genomically integrated *Zizania* DNA. The changes in both methylation and expression in the two introgression lines were stably inherited through generations, and may have contributed to some of the phenotypic novelties, including plant stature, disease-resistance and changes in yield components, that are characteristic of these lines [sic]” (Liu, et al. 2004).

5.3.23.2 This is yet another justification for imposing the special condition that no hybrids formed with LY038 are considered approved even if the LY038 event is approved.

(6) Substantial equivalence data.

6.1 Compositional

6.1.1. In MSL-19172 the Applicant compares the composition of LY038 to the negative segregant control, LY038(-). The test and control substance were grown at five replicated field sites in the U.S. In addition, four different commercially available conventional maize lines were grown at each of the five sites, for a total of twenty different conventional lines replicated once. Forage and grain samples were collected and analyzed for nutritional components.

6.1.2. The Applicant concluded from this study that “LY038 is considered to be compositionally equivalent to conventional maize except for the intended increases” in high lysine corn.

6.1.3. It is not clear to us how the Applicant arrived at this conclusion. We outline the reasons for this lack of clarity below.

6.1.4. Of the 396 comparisons made between LY038 and LY038(-), 103 were statistically

different (see Figure 4). This would mean that expression of cDHDPS, the effects of introducing I-DNA into the pre-LY038 line, subsequent processing by Cre recombinase, or the variability between environments in which LY038 would be routinely cultivated has introduced a statistically significant difference in 26% of the characters measured between these sibling lines. In order to gain a sense of how significant this difference between such closely genetically matched lines is, the Applicant should provide an analysis of variance of individuals within each line grown under matched conditions simultaneously.

- 6.1.5. The Applicant argues that of these 103 statistically significant differences, 81 (see Figure 4) were still “within the 99% tolerance interval of the population of commercial references”.
 - 6.1.5.1 It is difficult to reconcile how differences in the composition of LY038 with its closest genetic match, LY038(-), can be dismissed because its composition is within the variance of commercial references that are genetically distant to LY038 [in comparison to LY038(-)] and to each other. This would argue that LY038 is very unlike LY038(-) because the two have become as dissimilar as either is to genetically distant lines of corn, and these differences could be due to a very different mixture of genetic and physiological factors that could only have arisen from the modification of LY038.
 - 6.1.5.2 Is the variance between unrelated lines so large that it dwarfs variances between related, but distinct, lines? If so, then these commercial lines are no better a control than LY038(-) which, as we already know, has 103 statistically significant differences with LY038.
 - 6.1.5.3 Is the variance between environments more important than the variance between lines? If so, then the 99% tolerance interval is meaningless unless we know all the kinds of conditions that LY038 may be grown in and determine if the metabolic profile of LY038 in each of those environments is acceptable for human food.
- 6.1.6. Fourteen of the 22 differences (see Figure 4) that were outside the tolerance intervals were intended differences in lysine and free lysine, and differences in the associated lysine catabolite saccharopine in grain tissue. There is 100-times more saccharopine by weight in LY038 than in LY038(-) and at least 50-times more than in the reference controls. While differences in the production of saccharopine may have been anticipated, they are not intended and must be the subject of further studies (discussed below).
 - 6.1.6.1 The Applicant finds the α -amino adipic acid and saccharopine content of other common foods can be as high as or higher than levels in LY038. However consumption of these products is significantly less than the average consumption of maize. Based on United States per capita maize consumption (0.52 g/kg BW/day), the mean consumption of saccharopine is estimated to be 290 μ g/kg BW/day. This is more than 15-times the mean consumption of saccharopine in food containing high levels of saccharopine (e.g. button mushroom: 19 μ g/kgBW/day). Even if the per capita maize consumption in Australia and New Zealand were less than half that of the United States, or

0.2 g/kg/day, the level of saccharopine from LY038 would be an estimated 109 µg/kg BW/day. This would be more than five times the mean consumption of saccharopine in food containing high levels of saccharopine. Therefore, contamination of the human food supply with LY038 could introduce consumers to saccharopine at unprecedented concentrations.

- 6.1.7. The remaining eight statistically significant differences between LY038 and LY038(-) that were also outside the 99% tolerance intervals were considered unimportant either because their test values fell within ranges reported historically or within ranges reported in the scientific literature or because the difference between the absolute values of the test range and the tolerance interval were very small. The difference in the experimental conditions yielding historical and literature values makes it impossible to assess their relevance to LY038. So there remain some statistically significant differences between LY038 and LY038(-) that also fall outside the 99% tolerance interval that, in our view, cannot be dismissed simply because “the difference in the range of these values from the tolerance interval of commercial references was very small”. Accepting this explanation would be to deny the relevance of the 99% tolerance interval. Should the Applicant wish to deny the relevance of the 99% tolerance interval, then the 103 statistically significant differences between LY038 and LY038(-) must be accepted as indicating compositional non-equivalence.
- 6.1.8. We could not find measurements for asparagine and glutamine levels, separate from combined levels of aspartate + asparagine and glutamate + glutamine, in LY038. This concerns us because of their importance in potentially generating unwanted products by the Maillard reaction during cooking or processing. The Authority should insist that these amino acids be measured and related to the tolerance intervals of standard reference lines grown under matched conditions simultaneously.
- 6.1.9. Recent work has shown that “NMR combined with chemometrics and univariate statistics can successfully trace even small differences in metabolite levels between plants” (Le Gall, et al. 2003). This technique has been successfully tested using modified tomatoes. These findings make obsolete the observation in several recent review articles that metabolomics is still an uncertain science for assessing risk, and we recommend that this analysis be supplied to the Authority by the Applicant.

6.2 Informational

- 6.2.1. There are significant effects of RNA and DNA that are not measured through a description of the average content of ribo- and deoxyribo-nucleotides, nor even through the average content of polymers. Small RNA molecules, on the order of <30 nucleotides, for example, are potent gene regulators, giving rise to phenomena such as RNA interference (Cogoni and Macino 2000, Hannon 2002).
- 6.2.2. Microarray descriptions should be capable of detecting novel RNA species in the modified plant, with the RNA source being the plant grown under a variety of relevant field conditions. The microarray should comprehensively represent the

genomes of the cultivar of maize modified and unmodified. Since LY038 may be found in food, variant RNAs should be screen using a microarray for the human genome.

- (7) Measures of allergenicity characteristics, acute oral toxicity, and broiler growth are either too poorly described to meet our minimum standards for evaluation or are inadequate to meet our minimum standards for confidence in safety.

7.1 Allergenicity

- 7.1.1. The value of report MSL-18676, which purports to assess the potential allergenicity using a simulated gastrointestinal digestion assay, rests on the conclusion that *E. coli*-produced cDHDPS is in all relevant ways identical to *in planta*-produced cDHDPS. The Authority should note that in previous sections of this submission we have raised serious doubts about this conclusion. Besides, the Applicant should be using digestibility data to demonstrate that *in planta*-produced protein is equivalent to *E. coli*-produced cDHDPS using protein from both sources. All novel proteins in LY038 should be included in this comparison (not just the purified cDHDPS).
- 7.1.2. The FAO/WHO would seem to agree with us. They state that “the expressed protein should be assessed in its principal edible form under identical pepsin degradation conditions to those used to examine the expressed protein” (FAO/WHO 2001, p. 12). The Applicant only tested the latter, i.e. expressed protein isolated from *E. coli*.
- 7.1.3. There is no apparent justification for not using *in planta*-produced cDHDPS.
- 7.1.3.1 *In planta*-produced material was available because it was used in MSL-18585.
- 7.1.3.2 Using *in planta*-produced cDHDPS is the most straightforward and transparent way to conduct this study.
- 7.1.3.3 The Authority may recall from the NZIGE submission on application A524 (Food derived from herbicide-tolerant wheat MON 71800, subsequently withdrawn by the Applicant), comparability between the digestibility of the protein (CP4 EPSPS) produced either *in planta* or in *E. coli* could only be achieved when the Applicant used significantly different protocols. Without the results of a digestibility study using *in planta*-produced cDHDPS, the Authority cannot make a responsible determination of how well the *E. coli*-produced cDHDPS represents *in planta*-produced cDHDPS.
- 7.1.4. While it is our considered opinion that this study should not substitute for a properly conducted digestibility analysis using *in planta*-produced cDHDPS, we will also assess MSL-18676 on its own merits.
- 7.1.5. Digestibility studies are essential because proteins that do not digest readily can be allergens, as the Applicant’s researchers indicate (e.g. Bannon, et al. 2003).
- 7.1.6. The correlation between resistance to digestion by pepsin and a protein’s potential

to be an allergen is in doubt because some allergens are readily digested and some non-allergens are resistant to digestion (Fu, et al. 2002). Industry-independent observers note that “[l]ater work, however, cast some doubt on the usefulness of this test since few of all known food allergens demonstrate resistance to simulated gastric fluid (SGF-containing pepsin) or to simulated intestinal fluid (SIF) comprising pancreatin (a mixture of five enzymes: amylase, trypsin, lipase, ribonuclease, and protease). An explanation for the lack of correlation between SGF digestibility and nonallergenicity may be that both children and adults may have naturally or iatrogenically increased ventricular pH for extended periods” (Poulsen 2004).

- 7.1.7. The variability in human stomach pH is a key concern. It is known that infants have generally high stomach pH (approximately 3.0-4.0 and can be higher after food intake). The normal pH of an adult stomach is 2.0 but can raise to 5.0 after food intake (Schmidt, et al. 1995, Thomas, et al. 2004). Yet MSL-18676 reports analyses conducted at pH 1.2.
- 7.1.8. An important study compared pepsin-catalysed hydrolysis of whey proteins at pHs ranging from 2.0 to 4.0 and found that, at the pH of the infant stomach, antigenic epitopes were much more likely to survive and potentially pass into circulation (Schmidt, et al. 1995).
- 7.1.9. It should be noted that it will not be only adults that could be exposed to LY038 in their food supply. It would not be easy for parents of infants to avoid products derived from corn.
- 7.1.10. The relevance of measuring digestibility at pH1.2 is never made clear by the Applicant, beyond alluding to the recent demonstration that a group of industry and industry-led laboratories has established a common protocol to optimize reproducibility in these assays¹⁴ (see Thomas, et al. 2004). Whereas there may be virtue in establishing a standard, it remains unclear why the FAO/WHO protocol is not the standard nor why reproducibility is a greater virtue than using a pH relevant to conditions in the stomach during a meal, such as pH4-5 (Schmidt, et al. 1995, Thomas, et al. 2004).
- 7.1.11. The Applicant’s study also deviates from FAO/WHO recommendations on the pepsin to protein ratio.
 - 7.1.11.1 The Applicant used 10AU¹⁵ pepsin/μg *E. coli*-produced cDHDPS. Based on the experimentally observed activity of 3791AU per mg pepsin powder, there would be 2.64μg pepsin/10AU. FAO/WHO recommends 200μl of 0.32% (w/v) pepsin per 500μg protein (0.32% is 0.32mg/100ml, or 0.64μg in 200μl). The FAO/WHO recommendation is 0.001μg of pepsin/1μg cDHDPS, approximately 2,000 times less pepsin by weight compared to the amount used in MSL-18676.

¹⁴Citation mis-identified as Bannon *et al.* in MSL-18676 bibliography.

¹⁵Activity Units, or Units as defined by Applicant.

- 7.1.11.2 The weakness of the FAO/WHO protocol is that it does not control for variability in pepsin activities between studies. But an advantage of the FAO/WHO protocol is that it creates a more realistic ratio of pepsin to protein (Taylor 2003) in the relevant environment, the stomach (conditions that are not standardized for AU of pepsin). Which is the more important variable—pepsin activity variation between studies, or the different ratio of pepsin to protein in stomachs vs. *in vitro* assays—remains to be determined. In our opinion, it is the obligation of the Applicant to experimentally resolve this issue.
- 7.1.12. Probably the most important control for comparability between studies would be the use of a common set of control proteins, as recommended by the FAO/WHO. “Both known non-allergenic (soybean lipoxygenase, potato acid phosphatase or equivalent) and allergenic (milk beta lactoglobulin, soybean trypsin inhibitor or equivalent) food proteins should be included as comparators to determine the relative degree of the expressed proteins pepsin resistance” (p. 12). We could not find these controls in MSL-18676.
- 7.1.13. In the absence of such controls, we must agree with Taylor who says that even “a 10x ratio of pepsin to protein...is arguably ridiculous because *in vivo* protein would always exceed pepsin” (Taylor 2003). Certainly then, 2,000x pepsin is an excess that should be more carefully justified.
- 7.1.14. The Applicant should report digestibility measurements after processing. Nearly all native proteins are digestible using pepsin. Whether food proteins are digestible depends not just on their inherent digestibility, but also on the degree to which they have been modified during food processing (Meade, et al. in press). The extent to which cDHDPS expressed in maize and mDHDPS will be modified during food processing is unknown. Known differences in sequence (Mirwaldt, et al. 1995) and presumed differences in tertiary and quaternary structure (see above) are likely to affect the degree to which the protein is modified. This could influence proteolysis in the gut. It is entirely possible that cDHDPS would be less digestible post-processing, increasing its potential to elicit an immune response.
- 7.1.15. The digestion studies were submitted in part to reassure FSANZ and the public that the modified corn has low potential as a novel source of allergens. It is our view that these digestion studies were fundamentally flawed with regard to conclusions of allergenicity. Our argument is that:
- 7.1.15.1 the techniques used to assess the digestibility of cDHDPS event were inconsistent with international minimum standards;
 - 7.1.15.2 these standards were set by FAO/WHO, and FSANZ assessments are meant to be compliant with FAO/WHO principles;
 - 7.1.15.3 no studies were performed on *in planta*-produced protein or processed foods derived from LY038, making unintended effects creating potential allergens undetectable (Pusztai, et al. 2003).
- 7.1.16. We recommend that the Authority require the Applicant, at a minimum, to supply

data on the digestibility of the cDHDPS protein using a protocol compliant with the FAO/WHO standard and the recommendations of Pusztai *et al.* (Pusztai, et al. 2003).

- 7.1.17. The literature in the past several years has indicated that extrapolation from *in vitro* studies is no substitute of tests using animals (Pusztai, et al. 2003). For example, the Cry1Ab protein from genetically engineered corn survived digestion in the stomach of pigs and was detected by ELISA, immunochromatography and immunoblot in the intestine, despite it being shown to be highly digestible through the type of *in vitro* studies reported for *E. coli*-produced cDHDPS (Chowdhury, et al. 2003). Similarly, large fragments of the *cry1Ab* gene found in corn survived digestion and were detected in fecal material (Chowdhury, et al. 2003).
- 7.1.18. Interestingly, DNA and protein from the natural source (*Bacillus thuringiensis* subsp. *kurstaki*) was not detected in control pigs, indicating that the concentration of this material, or its structure, when present in feed corn differs from the material introduced into food through natural contamination by soil microorganisms. The differences are worthy of investigation. Many other studies also based on *in vivo* data of protein or DNA stability have made similar claims. It is our view that *in vivo* data from reliable studies will always be more trustworthy than extrapolations from *in vitro* data.
- 7.1.19. Furthermore, the Applicant should address possible effects of novel foods on the intestinal flora. *In vitro* studies cannot do this. Few if any *in vivo* studies have been designed to do this (aside from looking at microbial recombinants arising from transgenic DNA surviving digestion (Netherwood, et al. 2004)). Data are accumulating that soil flora can be affected by some forms of transgenic crops, such as Bt rice (Wu, et al. 2004). If soil flora are affected, then intestinal flora could be affected. The intestinal flora are critical to good health. Disease states arise from subtle shifts in population structure, not just the introduction of new pathogenic species (Berg 1995, Berg 1996).
- 7.1.20. In MSL-18744, the Applicant compared the primary amino acid structure of cDHDPS to known allergens using bioinformatics tools to identify any similarities between cDHDPS and confirmed allergens. Reassuringly, no significant matches were detected.
- 7.1.21. Bioinformatics tools have never been validated as comprehensively predictive of potential allergens. So such approaches are useful when they return a match rather than when they return no matches.
- 7.1.22. These types of tools are highly dependent on the quality of data provided to them. Search algorithms will not extrapolate the sequence space, for example, to potential polypeptide products arising from alternative splicing.
- 7.1.23. We have identified some peptide sequences that could arise from inclusion of parts of the rACT1 intron by alternative splicing for analysis. We analysed these peptides using Allermatch™ (Fiers, et al. 2004). While the results are not striking, this casual analysis retrieved a significant match to the known allergen Cla h 3

from *Cladosporium herbarum*. Since all alternative transcripts and peptides not properly processed for import into the chloroplast cannot be predicted in advance, the Applicant should produce algorithms that explore the sequence space of possible alternative transcripts and compare them to known allergens.

- 7.1.24. The Applicant should also provide evidence that novel proteins produced by LY038 pose no new risks due to a propensity to form aggregates as discussed in Part One.
- 7.1.25. A new resource for predicting the aggregation potential of proteins has recently become available (Fernandez-Escamilla, et al. 2004). This bioinformatics approach is based on a statistical mechanics algorithm called TANGO, a free web resource. It has been successfully trialled on 179 polypeptides from 21 different proteins and it successfully predicted the aggregation potential of up to 92% of the polypeptides.
- 7.1.26. The algorithm cannot be used to make quantitative comparisons of highly divergent polypeptides. Moreover, it is in theory less accurate with polypeptides over 40 residues. Nevertheless, it has demonstrated a qualitative efficacy in making predictions on longer polypeptides and provides guidance as to whether a protein has regions prone to seeding aggregation.
- 7.1.27. In our analysis of cDHDPS and mDHDPS, we found a different sequence landscape for aggregation potential (see Figure 5). These results suggest that high quantities of cDHDPS in the human diet may increase exposure to new forms of protein with properties different from mDHDPS. The safety of these species in food is unknown.

7.2 Acute oral toxicity and broiler feeding trials.

- 7.2.1. In MSL-18735 (acute oral toxicity), the Applicant fed 40 mice in two groups of 10 animals per sex, with either the test protein, cDHDPS, or a control protein, BSA. The target doses were 1000 mg/kg body weight. The test protein was cDHDPS isolated from *E. coli*. Each mouse received a single dose of the protein by gavage followed by *ad libitum* feeding on commercial feed for the 14 day trial. No significant weight changes were detected over the trial.
- 7.2.2. The first difficulty with this study is that the protein should have been sourced from LY038 rather than *E. coli*. This point has been made repeatedly by the scientific community (Pusztai, et al. 2003). The Applicant needs to address this and supply results of properly conducted studies before it is possible to make an informed decision on the application.
 - 7.2.2.1 Further, when this is done, toxicity tests on the whole crop are needed. “[C]ases where the composition of the whole crop has been changed significantly compared with the traditional counterpart¹⁶, or where there is a need to further investigate potential unintended side effects of the genetic

¹⁶Note that LY038 differs significantly in many ways from its traditional counterpart and, importantly, from its closest nontransgenic relative, as discussed in section 6.

modification, warrant additional toxicity testing” (Kok and Kuiper 2003, p. 440). As unintended side effects have been found in recent tests of other genetically engineered plants (Pusztai, et al. 2003), and 103 statistically significant differences were reported in MSL 19172, there is reason for additional toxicity testing here.

- 7.2.3. In any supplemental study, it is essential to weigh and observe the organs of the sacrificed animals. We recommend that the Authority disregard the Applicant’s claims on acute oral toxicity until MSL-18375 is supplemented with the information we believe is essential. “The biological, immunological, hormonal properties and allergenicity of” LY038 maize must be determined using the maize product and not surrogate sources such as *E. coli* (Pusztai, et al. 2003).
- 7.2.4. In MSL-18883 the Applicant compared the growth performance of broilers when fed diets containing LY038, LY038 x MON810, negative segregant control (LY038(-)), or four lots of commercial maize. No differences were found between broilers fed diets containing LY038 or LY038 x MON810 maize and broilers fed lysine-supplemented diets containing either control (LY038(-)) or commercial maize.
- 7.2.5. Adding lysine to the corn-diet of the broilers enhanced the adjusted weight gain considerably, as expected, but the growth of broilers given transgenic corn (GM) that produced higher amounts of lysine (LY038 and LY038 x MON810) was less dramatic during the first 21 days (see Figure 6). Broilers fed GM corn had significantly lower adjusted gain in the first 21 days relative to feed than groups fed conventional corn supplemented with similar amounts of lysine (p=0.008; t-test). This result suggests that there may be an unexpected and unexplained negative factor acting on broilers fed GM lysine-producing corn that prevented them from reaching the same growth rates as broilers fed conventional corn.
- 7.2.6. While no unintended effects were found in the broilers fed with LY038 maize, we recommend that the Authority seek blood tests and data on organ weights and visual observations.
- 7.2.7. We also were troubled by the apparent contamination of LY038 seed stock by MON810, a different transgenic line, reported in this study. Individual PCR analysis found that up to 20.5% of LY038 seed carried the MON810 event, or that up to 20.5% of the seed was MON810. This was also confirmed using an immunological detection method.
 - 7.2.7.1 The Applicant goes on to say that “the presence of the MON810 trait was considered as an ‘inert ingredient’ that would not impact the objectives and interpretation of this study” (MSL-18883, p. 23 of 165). In our view, the presence of substantial quantities of MON810 invalidates claims about LY038 because it has substantially diluted any concentration-dependent effects of the high lysine line.
 - 7.2.7.2 We do not at this stage trust that MSL-18883 delivers reliable assessments of LY038 effects on broilers. Coupled with the problems we discuss

immediately below, we recommend that the Authority dismiss study MSL-18883 for purposes of assessing safety.

- 7.2.8. There is another anomaly with the design of A549. Why were none of the commercial corn lines used in MSL-18883 included in MSL-19172 (compositional analysis)? Surely the most complete analysis would have used a set of lines common to both studies.
- 7.2.9. Strikingly, LY038 had higher levels of 16 of the 20 measured amino acids among the four commercial lines used as references in MSL-18883. The only amino acid (ignoring lysine) in which LY038 was not the highest or equal highest producer was proline (Table 1, Appendix 1 of MSL-18883). While we do not know how significant any individual measure is between LY038 and one of the commercial lines, it would seem an enormous coincidence if LY038 was the highest of the five lines in 16 amino acids by chance.
- 7.2.10. This raises questions about the design of MSL-18883, which appears flawed by chance or design to produce the greatest growth effect on the broilers.
- 7.2.11. And it raises other important questions. How would including these four commercial lines, each with amino acid levels clearly on the lower end of the spectrum, have influenced the 99% tolerance intervals calculated in MSL-19172? Our brief look suggests that the commercial lines in MSL-18883 *frequently* fall outside the 99% tolerance intervals in MSL-19172 (Table 1) and therefore would likely lower the interval. In many cases, LY038 was closer to the upper end of the tolerance interval. Including the four commercial varieties used in MSL-18883 in MSL-19172 might have revealed many more statistically significant differences in composition between LY038 and conventional corn.
- 7.2.12. We recommend that a compositional analysis that includes the four commercial varieties used in MSL-18883 be requested by the Authority.

Table 1. Amino acid analysis for LY038 and commercial corn.

Component	Tolerance Interval MSL-19172 ¹	Average MSL-18883 ^{2,3}	LY038 ⁴
Glutamic Acid	16.76-22.36	14.95	23.20
Leucine	10.15-15.62	9.28	15.10
Methionine	1.54-2.41	1.63	2.55
Phenylalanine	4.49-5.68	3.70	5.74
Threonine	2.73-3.62	2.76	3.87

¹Table 14. MSL-19172

²of four commercial references in Table 1, Appendix 1 MSL-18883

³numbers in bold are outside 99% tolerance interval measured in MSL-19172

⁴as reported in MSL-18883

7.3 Additional data needed.

- 7.3.1. Giroux *et al.* (1999) measured cholesterol concentration in serum and lipoprotein,

and liver phospholipids, in their feeding study on rabbits (Giroux, et al. 1999), finding significant differences in rabbits fed diets enriched in lysine or lysine and methionine. We could find no similar measurement in MSL-18735, or in the summary of the LY038 feeding study (Monsanto Australia Limited 2004, p. 103)¹⁷, and recommend that this type of data be submitted to the Authority.

- 7.3.2. Renwick (2004) lists the minimum essential characteristics of complete amino acid toxicity safety tests, which we reproduce in Table 2 (Renwick 2004). “Data from studies on humans may be of particular importance in the risk assessment of amino acids to confirm the absence of reversible adverse effects detected in animal studies (tolerability studies) or to define the potential human variability in biodisposition” (Renwick 2004, p. 1618S). Chronic testing is essential for establishing the allowable daily dose, however Renwick suggests that for amino acids “short-term and subchronic repeat-dose studies such as 90-d or 6-mo studies are of greater relevance...*It is now widely recognized that studies of 6- and 9-mo duration in rodents and nonrodents are sufficient for identification of possible hazards except cancer* and for dose-response assessment of noncancerous effects [emphasis added]” (Renwick 2004, p. 1619S).
- 7.3.3. The Authority should specifically consider requesting that the Applicant use the promising pig intestinal model for assessing amino acid toxicity (Baracos 2004). These models show promise for assessing the “maximal gut capacity to deal with amino acid insults under different physiological conditions” (Baracos 2004, p. 1658S). “The downregulation of intestinal protein degradation by amino acids contributes to controlling amino acid entry into free pools from proteolysis in the function of the dietary supply. Our models can be used to identify maximal response and the amino acids most potent in eliciting this change...This approach has application in studies to define the upward limits of the adaptive regulation of intestinal amino acid catabolism in response to high amino acid doses” (Baracos 2004, p. 1658S).
- 7.3.4. Cancer and other potential harms require lifetime feeding studies. Not all potential carcinogens or co-carcinogens may be related to the amino acids *per se*, but due to other changes in the GMO. That material must be used in such studies (Pryme and Lembcke 2003, Pusztai, et al. 2003).
- 7.3.5. We recommend that the Applicant provide a valid subchronic toxicity study of a minimum of 6 months duration.
- 7.3.6. We recommend that the Authority require toxicity testing commensurate with the recommendations of Table 2.

¹⁷Primary data was not released to us.

Table 2. Characteristics of amino acid toxicity safety tests.

Toxicity	Needed testing
Acute	Usually single-dose study
Short-term	Repeated doses for 14-28 days in actively growing animals (starting weight 80 grams for rats)
Subchronic	Repeated doses for 6-9 months (informs parameters for subsequent chronic toxicity study)
Chronic	Repeated doses for 2 years in rodents; 3 years rats
Reproductive	Dosing before, during and after gestation
Intestinal	Pig model

Part Three: Assessment of Impact Analysis

(8) Jurisdictional issues

- 8.1 Lack of clarity regarding the impacts relevant to the Analysis.
 - 8.1.1. FSANZ jurisdiction covers the *food* supply, yet the focus of the Impact Analysis is on the implications of the use of LY038 as *animal feed*.
 - 8.1.2. This raises problems for the evaluation of the application. Because FSANZ's jurisdiction is food safety, it seems that responses to the IAR in general, and the Impact Analysis in particular, that point out difficulties with regard to the importation of LY038 *as feed* are likely to be set aside during the FSANZ process. This creates a Catch-22 for assessing the Impact Analysis, and, thus, the proposed amendment to the Code.
 - 8.1.3. We urge FSANZ to re-examine whether the Impact Analysis should in fact include costs and benefits of importing or not importing LY038 *as feed*. If it decides in favour of such inclusion, we urge it to take into account the additional costs and benefits we identify.
- 8.2 The Applicant's assurance that LY038 is intended only as animal feed may have coloured the composition of the application.
 - 8.2.1. Our understanding of the Food Code is that any genetically modified organism that is permitted into the human food supply is considered safe at any concentration or frequency at which the unmodified organism may be consumed—not safe only at or below certain concentrations or frequencies of exposure.
 - 8.2.1.1 Even if contamination of the human food supply with LY038 were infrequent, or particular consignments had low concentrations, the concentration of the material may not be uniform in any food or consignment, resulting in some individuals experiencing higher than average exposures to the product. These individuals ought to be as safe in consuming the product as those who consume none of it.
 - 8.2.2. Our reading of A549 leaves us with the impression that the Applicant has assumed that a lower standard may be required for approval in this case, perhaps because there is no stated intent to have it become part of the human food supply. This is suggested by:
 - 8.2.2.1 The level of detail and strength of replication are much less for A549 than in some other applications, such as A524, and significantly decrease the potential for the Applicant to discover an unanticipated hazard. For example:
 - 8.2.2.2 MSL18365 adds to the volume of work submitted in support of the application, but fails to include the obvious controls, mDHDPS and *in planta*-produced cDHDPS, for its conclusions.
 - 8.2.2.3 The Applicant purified *in planta* cDHDPS using only one type of isolation

- scheme whereas in other applications this approach was augmented using immunological purifications techniques.
- 8.2.2.4 In contrast to A524, A549 lacks a digestibility comparison between *E. coli*-produced and *in planta*-produced protein.
 - 8.2.2.5 In contrast to A524, A549 lacks data on human immune responses.
 - 8.2.2.6 In the Applicant's discussion of a feeding study that was not a part of the materials released by FSANZ for independent review (Monsanto Australia Limited 2004, p. 103), they say "This level of exposure far exceeds the estimated human dietary exposure resulting from the *inadvertent introduction* of Lysine maize LY038 into the human food chain [emphasis added]". Thus, in this case their safety margin relies upon a frequency and concentration of contamination that is low rather than on an assurance of safety *per se*.
 - 8.2.2.7 In the Acute Oral Toxicity Study of A549 (MSL-18735) the animals were dosed with only one concentration of the test protein (cDHDPS), compared to the same study in A524 where three different doses were tested.
- 8.3 The proposed amendment would permit not only accidental or 'minimal' contamination; it would legalise any quantity of LY038 corn in food. Therefore, LY038 must be found safe regardless of the current intention on the part of the Applicant or any other producer of LY038 or public agency to segregate the product from corn intended for the human food supply. **We urge the Authority to disregard the declared intention of the Applicant to segregate LY038 from the human food supply, and to ensure that the declared intention influence neither the standards of rigour expected of the application nor the analysis of the impacts of amending the Code.**

(9) Impact Analysis

- 9.1 The inclusion of costs and benefits to users of LY038 as animal feed
 - 9.1.1. While FSANZ is not responsible for the evaluation of GM animal feed imports, its Impact Analysis considers several costs and benefits derived *from using LY038 as animal feed*. Since the material provided does not explain why failure to amend the Food Code will prevent animal growers from accessing LY038 feed, we can only speculate.
 - 9.1.2. Is it feared that LY038 will contaminate human *food* after it is imported into Australia and New Zealand as *feed*? This would imply that the Australian and New Zealand food-industry practices of segregating imported feed grains from food intended for human consumption are inadequate. If the response to inadequate practices in another sector is to amend the Code, then we fail to solve the primary problem. The first priority of the Code is to protect the safety of the food supply. Amending the Code should not, in our view, become so commonplace that it is used as an easy way to avoid solving other kinds of problems. If such segregation practices are indeed inadequate, this in itself should be a major concern of FSANZ.
 - 9.1.3. If it is not feared that LY038 will contaminate the human food supply after its importation as feed, then it remains unclear how this regulatory decision could

directly impact upon the availability of animal feed in Australia and New Zealand.

- 9.1.4. Is it feared that if Australia and New Zealand do not agree to permit LY038 in their food supplies, the Applicant will no longer grow LY038 at all, and therefore it will not be available to anyone? If this is the argument, then it should be stated explicitly and subjected to FSANZ and public scrutiny. It seems highly unlikely that the ability of LY038 to access the Australia/New Zealand food market would decide its global fate. If the implication here is that the costs of segregating LY038 from human food are prohibitive to the producers of LY038, then it must be recognised that should the Code be amended, such costs would fall on those seeking GM-free food (and feed).
- 9.1.5. If, on the other hand, seeking approval in Australia/New Zealand is part of the Applicant's business strategy to exert pressure on other food authorities directly or through the WTO, then this, too, should be explicitly stated and subjected to public scrutiny. This strategy has costs for growers, consumers, citizens, and governments in Australia and New Zealand.
- 9.1.6. In sum, we see no evidence or argument to suggest that the impacts of this regulatory decision fall upon animal growers. The contamination and commingling under discussion relate to corn seed and food products imported from LY038-growing countries. It seems that the declared intention to use LY038 only as feed has resulted in the misidentification of the Affected Parties (by focusing on animal growers, their suppliers and customers). We believe that as a result FSANZ is not in fact in a position to claim, as it does in its Statement of Reasons for accepting the Application, that "there is no reason to believe that costs arising from such a variation to include food derived from corn line LY038 would outweigh the direct and indirect benefits to the community, Government or industry that would arise from the variation." The *relevant* direct and indirect costs and benefits of the variation have not yet been scoped.
- 9.1.7. Finally, in our view it would be impermissible to give weight to impacts related to the animal-feed market when the Applicant was not required to provide information related to the safety and management of LY038 as an animal feed and FSANZ has no jurisdiction to determine its safety or suitability in this form. The benefits and costs of the use of LY038 as animal feed are therefore included without the appropriate investigation or evaluation. **We are concerned that FSANZ will base its decision in part on alleged benefits of LY038 that are apparently irrelevant to the Application and that it is not equipped to evaluate.**
- 9.1.8. Since these feed-related statements have been included, however, it is necessary to appraise them, and we do so below.
- 9.2 FSANZ defines Regulatory Option 1 as "not amending the Code to approve the sale and use of food derived from corn line LY038." Here we comment on the potential costs and benefits of this Option.
- 9.3 "Industry: Cost to animal growers as a possible reduction in the variety of animal

feed products available. Cost to animal growers to source either segregated or non-GM feed.”

- 9.3.1. As noted above, the material does not clarify why failure to amend the Code will prevent animal growers from accessing LY038 feed.
- 9.3.2. It seems that these alleged costs would be incurred only as a result of overseas’ suppliers inability to implement the kinds of quality-control measures that are essential to the traceability and product-chain monitoring required by some of our major markets. FSANZ appears to be asking Australians and New Zealanders to bear the risks that these suppliers’ lack of quality control creates.
- 9.3.3. The inadvertent and occasional lapse in segregation leading to rejected consignments for human use would not amount to significant cost, certainly not above what should be routinely borne by those who benefit from the sale of LY038. Should this not be the case, then we question whether the segregation procedures are as effective as claimed. If they are not, then the characterization of the amounts of LY038 expected in the human food supply as “likely to be low” is misleading.
- 9.3.4. Assuming, as the Impact Analysis does, that this regulatory decision would impact upon the composition of the animal-feed supply, the Impact Analysis fails to note the benefits of Option 1 for animal growers wishing to avoid GM animal feed.
 - 9.3.4.1 Rather than a speculative “possible reduction”, this benefit is immediate and measurable, in that it would obviate these growers’ otherwise necessary additional costs and other obstacles to maintaining a GM-free feeding regime.
 - 9.3.4.2 The size of these benefits may be considerable. The New Zealand Ministry of Agriculture and Forestry has acknowledged that “views on the feeding of genetically modified grain to poultry are one potentially significant influence on the direction of the poultry meat industry” (Ministry of Agriculture and Forestry 2003), projecting that, for the pig meat industry, if New Zealand grain crops remain free of GM, “the industry may be able to establish a national pork brand of ‘raised on GM free grain’ and gain a comparative advantage over other pork exporting countries” (Ministry of Agriculture and Forestry 2003b). The anti-GM-feed consumer sentiment that would prompt such a move is gaining momentum. A consortium of supermarkets in Britain removed GM crop-fed meat from their house brands in 1999 after concerns were raised by consumers (Woolf 1999), with this trend spreading to supermarkets across Europe. This suggests significant benefits to those who can keep GM feed out of their animal production process. Measures that reduce obstacles for these animal growers should be appropriately recognized.
- 9.4 “Industry: Cost in terms of restricting innovation in food/crop production for both growers and other sectors of the food industry.”
 - 9.4.1. This alleged cost is speculative because no evidence has been presented that Option 1 would result in something as sweeping as “restricting innovation in food/crop

production”. One could as readily argue that Option 1 will encourage innovation—for example, ways of accomplishing the same nutritional goals through genomics-assisted conventional crop breeding or other alternative ways of delivering the alleged benefits of LY038, ways that could prove highly marketable in themselves.

9.4.1.1 As noted in Part One, there are high lysine mutants in maize that are not the product of genetic engineering and could potentially be an alternative to LY038.

9.4.2. The “cost in terms of restricting innovation in food/crop production” must be offset by the benefits of not imposing new costs and constraints on those in the food industry developing and marketing GM-free products.

9.5 Option 1: WTO issues

9.5.1. The Impact Analysis lists as costs of Option 1: “Government: Potential impact if considered inconsistent with WTO obligations but impact would be in terms of trade policy rather than in government revenue.” “Industry: Potential longer-term impact - any successful WTO challenge has the potential to impact adversely on food industry.”

9.5.2. Because it is no one’s intent to introduce LY038 into the human food supply, Option 1 would presumably require only infrequent rejection of a consignment due to LY038 contamination. Occasional rejection on these grounds cannot legitimately be considered a non-tariff trade barrier if exercised only when the product was not of the composition expected. Thus there is little reason to expect Option 1 to be considered inconsistent with WTO obligations.

9.5.3. Moreover, WTO agreements must make allowances for importing countries should producers routinely fail in their obligations to demonstrate adequate care in the production, distribution and processing of human food. No international agreement should be used to justify poor quality-control procedures.

9.5.4. If, however, the argument here is that any refusal to allow a product (or a GM product, or a GM product from the United States) into the food supply should be considered a cost because someone might consider it inconsistent with WTO obligations, this should be made explicit and subjected to public scrutiny. Such an approach is inconsistent with FSANZ’s three primary objectives under Section 10 of the Act: to protect the public’s health and safety; to provide adequate information relating to food to enable consumers to make informed choices; and to prevent misleading or deceptive conduct. We note that a significant proportion (two out of five) of the impacts considered in the analysis of Option 1 relate to putative WTO concerns.

9.6 In light of the considerations outlined above, we believe that the Impact Analysis overstates the costs and understates the benefits of Option 1.

9.7 FSANZ defines Regulatory Option 2 as “amend[ing] the Code to permit the sale and use of food derived from corn line LY038, with or without listing special conditions

in the Table to clause 2 of Standard 1.5.2.” Here we comment on the potential costs and benefits of this Option.

- 9.8 “Consumers: Possible benefit of lower prices for poultry and swine food products, to the extent that savings from animal production efficiencies are passed on.”
- 9.8.1. Again, it is unclear why allowing LY038 in food (not feed) would impact upon the price of poultry and pork.
- 9.8.2. However, as this is being posited, we would like to draw attention to the conditional language in this statement. As it seems no price details have been settled on by the Applicant, it is impossible to determine the economic advantage or disadvantage LY038 entails for the food industry and consumers, compared with conventional high lysine corn or lysine supplements. This proposed benefit should be underpinned by an economic analysis that compares the projected cost of LY038 with the cost of conventionally adding lysine.
- 9.8.3. Similarly, no evidence has been presented that the potential costs of using LY038 incurred by animal growers (such as those related to segregation and monitoring, intellectual property, or overall nutritional value of the feed) will not offset its potential economic advantages.
- 9.8.4. Further, no evidence has been presented that any cost savings that might result would in fact be passed on to consumers. This would depend on a range of (unassessed) factors, including, for example, the market positions of producers, processors, distributors, and retailers.
- 9.9 “Consumers: The amount of LY038 corn entering the food supply is likely to be low so the cost to consumers wishing to avoid GM food by a potential restriction of choice or products, or increased prices for non-GM food is likely to be low.”
- 9.9.1. This statement rests on the Applicant’s declared current intention to import LY038 only as animal feed. However, the proposed amendment to the Code will approve LY038 for human consumption. We contend that any decision that is premised on a mere intention, which will not be secured by the regulation itself, is not appropriate or acceptable regulatory practice.
- 9.9.2. The proposed amendment is itself likely to weaken the declared intention. Once LY038 is legal in the food supply, there would be little incentive to minimize contamination. The Applicant would be compelled to maintain such stringency only if the amendment were declined. The current intentions and segregation measures emphasized by the Applicant are therefore inconsequential and should not figure in the Impact Analysis.
- 9.9.3. In their discussion of the Broiler Growth Trial (MSL-18883), the Applicant revealed that their LY038 stocks were contaminated with MON810 (see section 7). This case demonstrates that even the seed producer has difficulty maintaining separate stocks and suggests that the measures used by the Applicant are less reliable than claimed, or that the cost of monitoring to achieve reliable segregation

is prohibitively high. Given that the Applicant has not prevented the substantial contamination of its seed stock, FSANZ should scrutinize the claim that LY038 will only infrequently and at low quantities contaminate human food supplies.

- 9.9.3.1 Should FSANZ recommend amending the Code for the event in LY038, then it is critical that threshold criteria be established in Column 2 of the Table to Clause 2 of Standard 1.5.2 indicating below which levels and frequency of contamination, and range of contaminated products, LY038 events would be seen as inadvertently contaminating the human food supply and what the consequences would be for contamination above these thresholds.
- 9.9.4. The increasing incidence of coeliac disease among Australian and New Zealand consumers suggests a potentially serious impact of the introduction of LY038 into the food supply. Coeliacs are allergic to specific wheat, barley, rye and oat proteins; corn is one of the few remaining staple grains they are able to eat. Should coeliacs develop an allergic response to LY038, even its adventitious presence in the food supply will have serious consequences for them. Incidence of the disease is estimated at 1:300 (Kennedy and Feighery 2000). Studies in Australia and New Zealand have confirmed equal or greater rates in local populations (e.g. Hovell, et al. 2001, Cook 2004). A thirty-year study of diagnoses in the Canterbury region found rising rates of the disease among both adults and children over that period. Adult prevalence is noted as 1:83 in the Christchurch area (Cook 2004). The special dietary requirements of this large section of the population reaffirm the importance of carefully screening the introduction of new ingredients to the food supply. The range of products identified by the Applicant as vulnerable to contamination further argues against the estimation of a “low” impact on consumers wishing to avoid GM food. The possibility that the proposed amendment may result in the elimination of more foods from coeliacs’ already strict eating plan should be considered a significant cost.
- 9.9.5. The statement implies that those wishing not to consume LY038 will bear the cost of avoiding LY038. There are three difficulties with this suggestion.
 - 9.9.5.1 First, only if the Code were amended to permit LY038 would it become the burden of the citizen to pay to avoid LY038. The status quo places the burden on those who benefit from the sale of the product. Thus, opting for Option 2 has a guaranteed impact on the citizen regardless of whether there is financial cost; Option 2 creates a new imbalance of power between consumer and producer.
 - 9.9.5.2 Second, the cost of demonstrating that a product is LY038-free will be just as high whether LY038 is a rare or a frequent contaminant of other corn-derived products. The testing is not simpler nor is it necessarily less rigorous or frequent. It will have to be conducted continually without certain knowledge of when or where the contamination will occur. Evidence should be provided for the assertion that the cost of avoiding LY038 will be low. Our internationally peer-reviewed work on monitoring does not lead us to this conclusion (Heinemann, et al. 2004).
 - 9.9.5.3 Third, since the Applicant’s case is that LY038 will only rarely contaminate

corn intended for human consumption, the cost of that assurance ought to be borne by the Applicant and/or others who benefit financially from the production of LY038, as is the case under Option 1. Thus, we think FSANZ has inappropriately underestimated the impact on the consumer of opting for Option 2.

9.10 “Government: Benefit that if LY038 were to inadvertently enter the human food supply, this application will ensure any corn imports from the United States comply with the Code. This would ensure that there is no potential for trade disruption on regulatory grounds.”

9.10.1. We find this statement troubling and inconsistent with the Authority's three primary objectives: to protect the public's health and safety; to provide adequate information relating to food to enable consumers to make informed choices; and to prevent misleading or deceptive conduct.

9.10.1.1 To regard as a benefit the facilitation (through removal of disincentives) of adventitious contamination of the food supply with (GM) animal feed cannot be regarded as protective of the public's health and safety, either in itself or through the precedent it sets.

9.10.1.2 “Ensur[ing] that there is no potential for trade disruption” requires measures that will simultaneously ensure that the public will not be provided with the information they need in order to make informed choices regarding LY038-contaminated food, as they will not be made aware of the presence of the contaminant (and at the same time, as we noted above, the proposed change to the Code makes such contamination more likely).

9.10.1.3 Increasing the likelihood that the public remains unaware of instances of adventitious contamination of the food supply (i.e., by removing the regulatory and trade dimensions that might draw attention to it) could *facilitate* misleading and deceptive conduct by the food industry, particularly by those who export to us.

9.10.2. If we were to take this alleged benefit to its logical conclusion, the Code would be amended to permit any and all food imports and contaminants thereof, or at least those from the United States, in order to minimize the overall “potential for trade disruption on regulatory grounds”. Such a position is in obvious and direct conflict with the Authority's primary objectives.

9.11 “Government: This decision may impact on monitoring resources as food derived from corn line LY038 will be required to be labelled as GM and may be required to be labelled as having altered characteristics.” “Industry: Possible cost to food industry as food derived from corn line LY038 will be required to be labelled as genetically modified and may be required to be labelled regarding its increased lysine levels.”

9.11.1. In contrast to many of the benefits of Option 2 suggested by the Impact Analysis, monitoring and labelling costs are immediate and certain, rather than speculative, impacts of the proposed amendment. The implications should therefore be more

carefully detailed, showing the extent to which the introduction of LY038 might impact already stressed resources with unique monitoring requirements to be applied to the full range of products the Applicant has identified as being at risk of contamination. The list is extensive, and it is reasonable to expect a significant impact on monitoring resources. Similarly, the costs of labelling will certainly impact upon the food industry.

- 9.11.2. While the frequency and amount of contamination are irrelevant to the standards of the scientific safety evaluation, they are not irrelevant to the Impact Analysis. When the cost to producers of GM-free products increases because they must monitor their sources for GM material, that cost is permanent and considerable irrespective of the frequency of contamination. GM-free producers cannot opt to monitor only when they know their product has been contaminated. New Zealand has learned numerous times in the past four years how difficult it is to detect GM material and to attempt to monitor it correctly (Heinemann, et al. 2004). That lesson is well documented in the international, peer-reviewed scientific literature.
- 9.11.3. A review of the economic impact of LY038 should also consider the costs involved in the post-launch monitoring recommended in the event of an amendment (see 3.6).
- 9.11.4. All of this argues that the costs, both to industry and to government, are more than just possibilities and that they are likely to be considerable.
- 9.12 “Industry: Possible benefit to animal growers in terms of a wider range of feed products. Benefit to importers and distributors of overseas feed products as the product range is extended.”
 - 9.12.1. Again, nowhere in the material provided is it explained why amendment of the Code is necessary in order to import LY038 animal feed.
 - 9.12.2. Increasing the feed products on the market is not necessarily a benefit for animal growers. We note that in a recent review commissioned by the New Zealand Food Safety Authority, the Poultry Industry Association of New Zealand “expressed strong concerns that there were insufficient controls on the importation of...raw materials that are ingredients for animal feed” (External Review Team 2004).
 - 9.12.3. This “possible benefit” must again be offset by the certain cost to growers who wish to avoid GM feed, and to distributors and exporters who wish to maintain GM-free product lines, in that the approval of this feed will increase the obstacles they face to maintain this standard.
- 9.13 FSANZ should also consider that New Zealand will ratify the Cartagena Protocol on Biosafety. This makes New Zealand’s international obligations different from Australia’s and means that there are additional significant impacts for New Zealand. For example, should some LY038 seed contaminate New Zealand feed corn crops, then New Zealand will be subject to special requirements for reporting on its own corn exports.

Conclusion

The submitter is a recognized research centre in biosafety with expertise in molecular biology and genetics in general and genetic engineering in particular, social and political science, biochemistry and protein science.

Having examined the application, our conclusion is that significant additional information should be provided by the Applicant before a decision is made on whether to amend the Australia New Zealand Food Code to allow food derived from LY038. There have been significant advances in biosafety and risk assessment science that are not consistently reflected in the standard of reporting in A549. The studies submitted in support of A549 do not consistently meet what we see as the standard of the science.

Having examined the Impact Analysis, our conclusion is that it is necessary to clarify *which* costs and benefits to which Affected Parties are appropriately included in an application to amend the Food Code. Further, we have identified a number of questionable inclusions, omissions, and assumptions, the result of which is an Impact Analysis that overstates the benefits and understates the costs of amending the Code. We have also raised *bona fide* issues of safety that have not been addressed by the Authority or the Applicant, which also increase the cost of amending the Code.

On the basis of the evidence and analysis provided thus far, amending the Food Code to permit the use of LY038 in food cannot be justified.

Respectfully submitted on behalf of all authors,



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Director

Bibliography

A549 Public Register

Monsanto Australia Limited (2004). Application to Food Standards Australia New Zealand for the inclusion of Lysine maize LY038 in Standard 1.5.2 – Food Derived from Gene Technology.

MSL-18365: Rice, E.A., S.A. Kapadia, C.M. Dalton, T.P Brown, R.S. Thoma, R.E. Hileman and J.D. Astwood. (2003). “Characterisation of the *E. coli*-produced *Corynebacterium glutamicum* Dihydrodipicolinate Synthase (cDHDPS) Protein”. An unpublished study conducted by Monsanto Company.

MSL-18585: Rice, E.A., S.A. Kapadia, R.S. Thoma and R.E. Hileman. (2003). “Characterisation of the cDHDPS Protein Purified from Grain of Lysine Maize LY038 and Assessment of the Physicochemical and Functional Equivalence of the Plant-Produced cDHDPS Protein and the *E. coli*-produced cDHDPS Protein”. An unpublished study conducted by Monsanto Company.

MSL-18676: Rice, E.A., S.A. Kapadia and R.E. Hileman. (2003). “Assessment of the *in vitro* Digestibility of the cDHDPS Protein in Simulated Gastric Fluid”. An unpublished study conducted by Monsanto Company.

MSL-18735: Kaempfe, T.A. (2003). “An Acute Oral Toxicity Study in Mice with *E. coli*-Produced cDHDPS Protein”. An unpublished study conducted by Monsanto Company.

MSL-18744: McCoy, R.L. and A. Silvanovich. (2003). “Bioinformatics Analysis of the cDHDPS Protein Expressed in Lysine Maize Event LY038 and LY049 Utilizing the AD4, TOXIN5 and ALLPEPTIDES Databases”. An unpublished study conducted by Monsanto Company.

MSL-18883: Taylor, M.L., Y. Hyun, G.F. Hartnell, M.A. Nemeth, K. Karunanandaa, B. George, K.C. Glenna and W.F. Heydens. (2003). “Sponsor Summary of Report for Study 02-01-72-16 (Comparison of Broiler Performance When Fed Diets Containing LY038, LY038 x MON810, Negative Segregant Control, or Commercial Maize)”. An unpublished study conducted by Monsanto Company.

MSL-19109: Mittanck, D.W., J.F. Rice, G.M. Palmer and S.E. Reiser. (2004). “Amended Report for MSL-17770: Molecular Analysis of Lysine Maize LY038”. An unpublished study conducted by Monsanto Company.

MSL-19172: Reynolds, T.L., M.A. Nemeth, J.D. Fuhrman, W.A. Trujillo and R. Sorbet. (2004). “Compositional Analysis of Forage and Grain Collected from Lysine Maize LY038 Grown in U.S. Field Trials in 2002”. An unpublished study conducted by Monsanto Company.

Other references

- Altuvia, S. and E. G. H. Wagner (2000). "Switching on and off with RNA." *Proc. Natl. Acad. Sci. USA* 97(18): 9824-9826.
- Azevedo, R. A. (2002). "Analysis of the aspartic acid metabolic pathway using mutant genes." *Amino Acids* 22: 217-230.
- Azevedo, R. A. and P. J. Lea (2001). "Lysine metabolism in higher plants." *Amino Acids* 20: 261-279.
- Bannon, G., T.-J. Fu, et al. (2003). "Protein digestibility and relevance to allergenicity." *Environ. Health Pers.* 111: 1122-1124.
- Baracos, V. E. (2004). "Animal models of amino acid metabolism: A focus on the intestine." *J. Nutr.* 134, 6S: 1656S-1659S.
- Baulcombe, D. (2004). "RNA silencing in plants." *Nature* 431: 356-363.
- Berg, R. D. (1995). "Bacterial translocation from the gastrointestinal tract." *Trends Microbiol.* 3(4): 149-154.
- Berg, R. D. (1996). "The indigenous gastrointestinal microflora." *Trends Microbiol.* 4(11): 430-435.
- Bessler, C., J. Schmitt, et al. (2003). "Directed evolution of a bacterial {alpha}-amylase: Toward enhanced pH-performance and higher specific activity." *Protein Sci* 12(10): 2141-2149.
- Blickling, S., H. G. Beisel, et al. (1997). "Structure of dihydropicolinate synthase of *Nicotina sylvestris* reveals novel quaternary structure." *J. Mol. Biol.* 274: 608-621.
- Boutla, A., K. Kalantidis, et al. (2002). "Induction of RNA interference in *Caenorhabditis elegans* by RNAs derived from plants exhibiting post-transcriptional gene silencing." *Nucleic Acids Res.* 30: 1688-1694.
- Bucciantini, M., E. Giannoni, et al. (2002). "Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases." *Nature* 416: 507-511.
- Burke, Z. D., T. Wells, et al. (2000). "Use of a cryptic splice donor site in the chloramphenicol acetyltransferase (CAT)-SV40 small-t antigen cassette generates alternative transcripts in transgenic rats." *Transgen. Res.* 9: 67-70.
- Cartegni, L., S. L. Chew, et al. (2002). "Listening to silence and understanding nonsense: exonic mutations that affect splicing." *Nat. Rev. Genet.* 3: 285-298.
- Castagna, A., N. Campostrini, et al. (2002). "Comparative two-dimensional mapping of prion protein isoforms in human cerebrospinal fluid and central nervous system." *Electrophor.* 23: 339-346.

- Chamruspollert, M., G. M. Pesti, et al. (2002). "Dietary interrelationships among arginine, methionine, and lysine in young broiler chicks." *Br. J. Nutr.* 88: 655–660.
- Chiti, F., N. Taddei, et al. (2000). "Mutational analysis of the propensity for amyloid formation by a globular protein." *EMBO J.* 19(7): 1441-1449.
- Chowdhury, E. H., H. Kuribara, et al. (2003). "Detection of corn intrinsic and recombinant DNA fragments and CryIAb protein in the gastrointestinal contents of pigs fed genetically modified corn Bt11." *J. Anim. Sci.* 81: 2546-2551.
- Cogoni, C. and G. Macino (2000). "Post-transcriptional gene silencing across kingdoms." *Curr. Opin. Genet. Develop.* 10: 638-643.
- Cook, B., Oxner, R., Chapman, B., Whitehead, M. & Burt, M. (2004). "A thirty-year (1970-1999) study of coeliac disease in the Canterbury region of New Zealand." *New Zeal. Med. J.* 117(1189).
- Denli, A. M. and G. J. Hannon (2003). "RNAi: an ever-growing puzzle." *Trends Biochem. Sci.* 28: 196-201.
- Dobson, C. M. (2001). "The structural basis of protein folding and its links with human disease." *Phil. Trans. R. Soc. Lond. B.* 356: 133-145.
- Einspanier, R., A. Klotz, et al. (2001). "The fate of forage plant DNA in farm animals: a collaborative case-study investigating cattle and chicken fed recombinant plant material." *Eur. Food Res. Technol.* 212(2): 129-134.
- Ellis, R. J. and J. T. Pinheiro (2002). "Danger - misfolding proteins." *Nature* 416: 483-484.
- External Review Team (2004). Strategic review of regulatory arrangements controlling the importation of foods and food-related products into New Zealand. Wellington, New Zealand Food Safety Authority.
- FAO/WHO (2001). Allergenicity of Genetically Modified Foods. Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology. Rome, Food and Agriculture Organisation of the United Nations.
- Faugeron, G. (2000). "Diversity of homology-dependent gene silencing strategies in fungi." *Curr. Opin. Microbiol.* 3: 144-8.
- Fayle, S. E. and J. A. Gerrard (2002). *The Maillard Reaction*. London, Royal Society of Chemistry.
- Fernandez-Escamilla, A.-M., F. Rousseau, et al. (2004). "Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins." *Nat. Biotechnol.* 22: 1302-1306.
- Fiers, M. W. E. J., G. A. Kleter, et al. (2004). "Allermatch™, a webtool for the prediction

of potential allergenicity according to current FAO/WHO Codex alimentarius guidelines." *BMC Bioinformatics* 5: 133-138.

Forsman, A., D. Ushameckis, et al. (2003). "Uptake of amplifiable fragments of retrotransposon DNA from the human alimentary tract." *Mol. Gen. Genomics* 270(4): 362-8.

FSANZ (2004). Initial Assessment Report: Application A549 Food Derived from High Lysine Corn LY038.

Fu, T. J., U. R. Abbott, et al. (2002). "Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluids-a comparative study." *J. Agric. Food Chem.* 50: 7154-7160.

Giroux, I., E. M. Kurowska, et al. (1999). "Role of dietary lysine, methionine, and arginine in the regulation of hypercholesterolemia in rabbits." *J. Nutr. Biochem.* 10(3): 166-171.

Graveley, B. R. (2001). "Alternative splicing: increasing diversity in the proteomic world." *Trends Genet.* 17(2): 100-107.

Gura, T. (2000). "A silence that speaks volumes." *Nature* 404(6780): 804-808.

Hannon, G. J. (2002). "RNA interference." *Nature* 418(6894): 244-251.

Heilmann, I., M. S. Pidkowich, et al. (2004). "Switching desaturase enzyme specificity by alternate subcellular targeting." *Proc. Natl. Acad. Sci. USA* 101(28): 10266-10271.

Heinemann, J. A., A. D. Sparrow, et al. (2004). "Is confidence in monitoring of GE foods justified?" *Trends Biotechnol.* 22: 331-336.

Hirotsune, S., N. Yoshida, et al. (2003). "An expressed pseudogene regulates the messenger-RNA stability of its homologous coding gene." *Nature* 423: 91-96.

Hohlweg, U. and W. Doerfler (2001). "On the fate of plant or other foreign genes upon the uptake in food or after intramuscular injection in mice." *Mol. Gen. Genomics* 265: 225-233.

Hovell, C., J. Collett, et al. (2001). "High prevalence of coeliac disease in the Busselton population: a case for screening?" *Med. J. Australia* 175: 247-250.

Jackson, A. L., S. R. Bartz, et al. (2003). "Expression profiling reveals off-target gene regulation by RNAi." *Nat. Biotechnol.* 21: 635-637.

Jackson, A. L. and P. S. Linsley (in press). "Noise amidst the silence: off-target effects of siRNAs?" *Trends Genet.*

Kennedy, N. P. and C. Feighery (2000). "Clinical features of coeliac disease today." *Biomed Pharmacother.* 54: 373-380.

Klotz, A., J. Mayer, et al. (2002). "Degradation and possible carry over of feed DNA

- monitored in pigs and poultry." *Eur. Food Res. Technol.* 214(4): 271-275.
- Kohli, A., S. Griffiths, et al. (1999). "Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination." *Plant J.* 17: 591-601.
- Kohli, A., R. M. Twyman, et al. (2003). "Transgene integration, organization and interaction in plants." *Pl. Mol. Biol.* 52: 247-258.
- Kok, E. J. and H. A. Kuiper (2003). "Comparative safety assessment for biotech crops." *Trends Biotechnol.* 21: 439-444.
- Le Gall, G., I. J. Colquhoun, et al. (2003). "Metabolite profiling of Tomato (*Lycopersicon esculentum*) using ¹H NMR spectroscopy as a tool to detect potential unintended effects following a genetic modification." *J. Agric. Food Chem.* 51: 2447-2456.
- Liu, Z., Y. Wang, et al. (2004). "Extensive alterations in DNA methylation and transcription in rice caused by introgression from *Zizania latifolia*." *Pl. Mol. Biol.* 54: 571-582.
- Makarevitch, I., S. K. Svtashev, et al. (2003). "Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment." *Pl. Mol. Biol.* 52: 421-432.
- Manzi, A. E., K. Norgard-Sumnicht, et al. (2000). "Exploring the glycan repertoire of genetically modified mice by isolation and profiling of the major glycan classes and nano-NMR analysis of glycan mixtures." *Glycobiology* 10(7): 669-689.
- Meade, S. J., E. A. Reid, et al. (in press). Amino acid and Protein Modifications due to the Processing and Storage of Foods - Implications for Amino Acid Analysis and Bioavailability. *Special Edition of AOAC International*. P. Moughan.
- Meister, G. and T. Tuschl (2004). "Mechanisms of gene silencing by double-stranded RNA." *Nature* 431: 343-349.
- Mello, C. C. and D. Conte Jr. (2004). "Revealing the world of RNA interference." *Nature* 432: 338-342.
- Ministry of Agriculture and Forestry (2003). Situation and Outlook for New Zealand Agriculture and Forestry 2003-Poultry Meat.
- Ministry of Agriculture and Forestry (2003b). Situation and Outlook for New Zealand Agriculture and Forestry 2003-Pig Meat.
- Mirwaldt, C., I. Korndorfer, et al. (1995). "The crystal structure of dihydrodipicolinate synthase from *Escherichia coli* at 2.5 Å resolution." *J. Mol. Biol.* 246: 227-239.
- Mochizuki, K. and M. A. Gorovsky (2004). "Small RNAs in genome rearrangement in

- Tetrahymena." *Curr. Opin. Genet. Develop.* 14: 181-187.
- Mottram, D. S., B. L. Wedzicha, et al. (2002). "Acrylamide is formed in the Maillard reaction." *Nature* 419: 448-449.
- Netherwood, T., S. M. Martín-Orúe, et al. (2004). "Assessing the survival of transgenic plant DNA in the human gastrointestinal tract." *Nat. Biotechnol.* 22: 204-209.
- Norregaard Jensen, O. (2004). "Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry." *Curr. Opin. Chem. Biol.* 8(1): 33-41.
- Ohnishi, A. and K. Takano (2004). "Amyloid fibrils from the viewpoint of protein folding." *CMLS Cell. Mol. Life Sci.* 61: 511-524.
- Palka-Santini, B. Schwarz-Herzke, et al. (2003). "The gastrointestinal tract as the portal of entry for foreign macromolecules: fate of DNA and proteins." *Mol. Gen. Genomics* 270: 201-215.
- Pepys, M. B. (2001). "Pathogenesis, diagnosis and treatment of systemic amyloidosis." *Phil. Trans. R. Soc. Lond. B.* 356: 203-211.
- Pfeffer, A., R. Schubbert, et al. (1999). "Integrated viral genomes can be lost from adenovirus type 12-induced hamster tumor cells in a clone-specific, multistep process with retention of the oncogenic phenotype." *Virus Res.* 59: 113-127.
- Poulsen, L. K. (2004). "Allergy assessment of foods or ingredients derived from biotechnology, gene-modified organisms, or novel foods." *Mol. Nutr. Food Res.* 48: 413-423.
- Pryme, I. F. and R. Lembcke (2003). "In vivo studies on possible health consequences of genetically modified food and feed - with particular regard to ingredients consisting of genetically modified plant materials." *Nut. Health* 17: 1-8.
- Pusztai, A., S. Bardocz, et al. (2003). Genetically modified foods: potential human health effects. *Food Safety: Contaminants and Toxins*. J. P. F. D'Mello. CAB International. 347-371.
- Rang, A., B. Linke, et al. (in press). "Detection of RNA variants transcribed from the transgene in Roundup Ready soybean." *Eur. Food Res. Technol.*
- Renwick, A. G. (2004). "Establishing the upper end of the range of adequate and safe intakes for amino acids: A toxicologist's viewpoint." *J. Nutr.* 134, 6S: 1617S-.
- Rudd, P. M. and R. A. Dwek (1997). "Glycosylation: heterogeneity and the 3D structure of proteins." *Crit. Rev. Biochem. Mol. Biol.* 32: 1-100.
- Rudd, P. M., T. Endo, et al. (1999). "Glycosylation differences between the normal and pathogenic prion protein isoforms." *Proc. Natl. Acad. Sci. USA* 96(23): 13044-13049.

Scacheri, P. C., O. Rozenblatt-Rosen, et al. (2004). "Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells." *Proc. Natl. Acad. Sci. USA* 101(7): 1892-1897.

Schilter, B. and A. Constable (2002). "Regulatory control of genetically modified (GM) foods: likely developments." *Toxicol. Lett.* 127: 341-349.

Schmidt, D. G., R. J. G. M. Meijer, et al. (1995). "Raising the pH of the pepsin-catalysed hydrolysis of bovine whey protein increases the antigenicity of the hydrolysates." *Clin. Exp. Allergy*. 25: 1007-1017.

Schmucker, D., J. C. Clemens, et al. (2000). "Drosophila Dscam Is an Axon Guidance Receptor Exhibiting Extraordinary Molecular Diversity." *Cell* 101(6): 671-684.

Schubbert, R., U. Hohlweg, et al. (1998). "On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus." *Mol. Gen. Genet.* 259: 569-576.

Schubbert, R., C. Lettmann, et al. (1994). "Ingested Foreign (phage M13) DNA Survives Transiently in the Gastrointestinal Tract and Enters the Bloodstream of Mice." *Mol. Gen. Genet.* 242: 495-504.

Schubbert, R., D. Renz, et al. (1997). "Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA." *Proc. Natl. Acad. Sci. USA* 94(3): 961-966.

Semizarov, D., L. Frost, et al. (2003). "Specificity of short interfering RNA determined through gene expression signatures." *Proc. Natl. Acad. Sci. USA* 100: 6347-6352.

Srivastava, V. and D. W. Ow (2003). "Rare instances of Cre-mediated deletion product maintained in transgenic wheat." *Pl. Mol. Biol.* 52: 661-668.

Stadler, R. H., I. Blank, et al. (2002). "Acrylamide from Maillard reaction products." *Nature* 419: 449.

Svitashev, S. K., W. P. Pawlowski, et al. (2002). "Complex transgene locus structures implicate multiple mechanisms for plant transgene rearrangement." *Plant J.* 32(4): 433-445.

Taylor, S. L. (2003). "Comment of Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluids-a comparative study." *J. Agric. Food Chem.* 51: 5183-5184.

Tchurikov, N. A., L. G. Chistyakova, et al. (2000). "Gene-specific Silencing by Expression of Parallel Complementary RNA in Escherichia coli." *J. Biol. Chem.* 275(34): 26523-26529.

Thomas, K., M. Aalbers, et al. (2004). "A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins." *Regul.*

Toxicol. Pharm. 39(2): 87-98.

Timmons, L., D. L. Court, et al. (2001). "Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*." *Gene* 263: 103-112.

Van den Steen, P., P. M. Rudd, et al. (1998). "Concepts and Principles of O-Linked Glycosylation." *Crit. Rev. Biochem. Mol. Biol.* 33: 151-208.

Vlassara, H., W. Cai, et al. (2002). "Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy." *Proc. Natl. Acad. Sci. USA* 99(24): 15596-15601.

Volchkov, V. E., V. A. Volchkova, et al. (2001). "Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity." *Science* 291: 1965-1969.

Weld, R., J. Heinemann, et al. (2001). "Transient GFP expression in *Nicotiana glumbaginifolia* suspension cells: the role of gene silencing, cell death and T-DNA loss." *Pl. Mol. Biol.* 45: 377-385.

Wilusz, C. J., W. Wang, et al. (2001). "Curbing the nonsense: the activation and regulation of mRNA surveillance." *Genes Dev.* 15(21): 2781-2785.

Windels, P., I. Taverniers, et al. (2001). "Characterisation of the Roundup Ready soybean insert." *Eur. Food Res. Technol.* 213: 107-112.

Woolf, M. (1999). Supermarkets join forces on GM animal feed. *Sunday Independent*. London, 13 June 1999.

Wu, W., Q. Ye, et al. (2004). "Effect of straws from Bt-transgenic rice on selected biological activities in water-flooded soil." *Eur. J. Soil Biol.* 40: 15-22.

Zhao, Z., Y. Cao, et al. (2001). "Double-Stranded RNA Injection Produces Nonspecific Defects in Zebrafish." *Develop. Biol.* 229: 215-223.

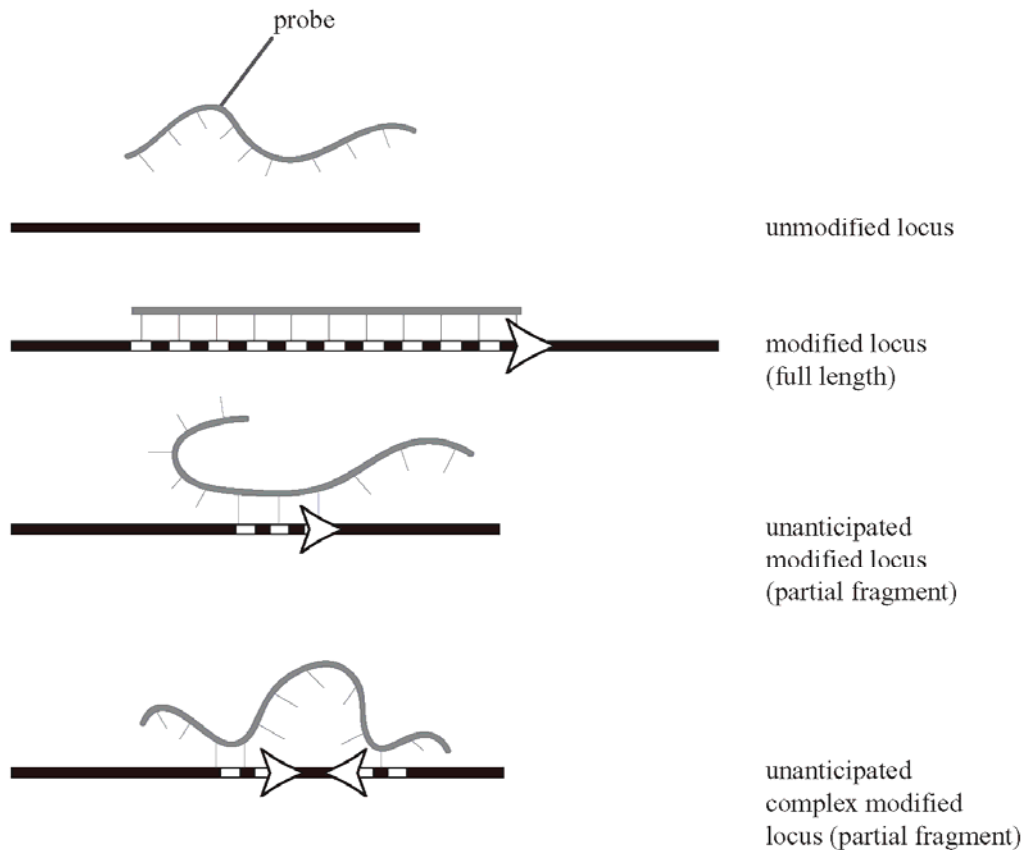


Figure 1

Examples of integration events that may lead to false negative results using long probes. Compared to controls (usually fully intact, full length plasmid DNA identical to the probe) and full length insertions, partial fragments appear as ghostly or light bands since the combination of the stringency in washes and the efficiency of binding at these sites would act to reduce the amount of probe retained. Below some threshold number of base pairs between probe and target DNA, the Southern would not detect the insert. Nevertheless, each insert is relevant to risk identification. The minimum standard in reporting should be: 1. supply controls showing size detection limits (i.e. determine the threshold for returning a positive result) at the same stringency used to conclude the absence of an insert; 2. sequence all ghost bands.

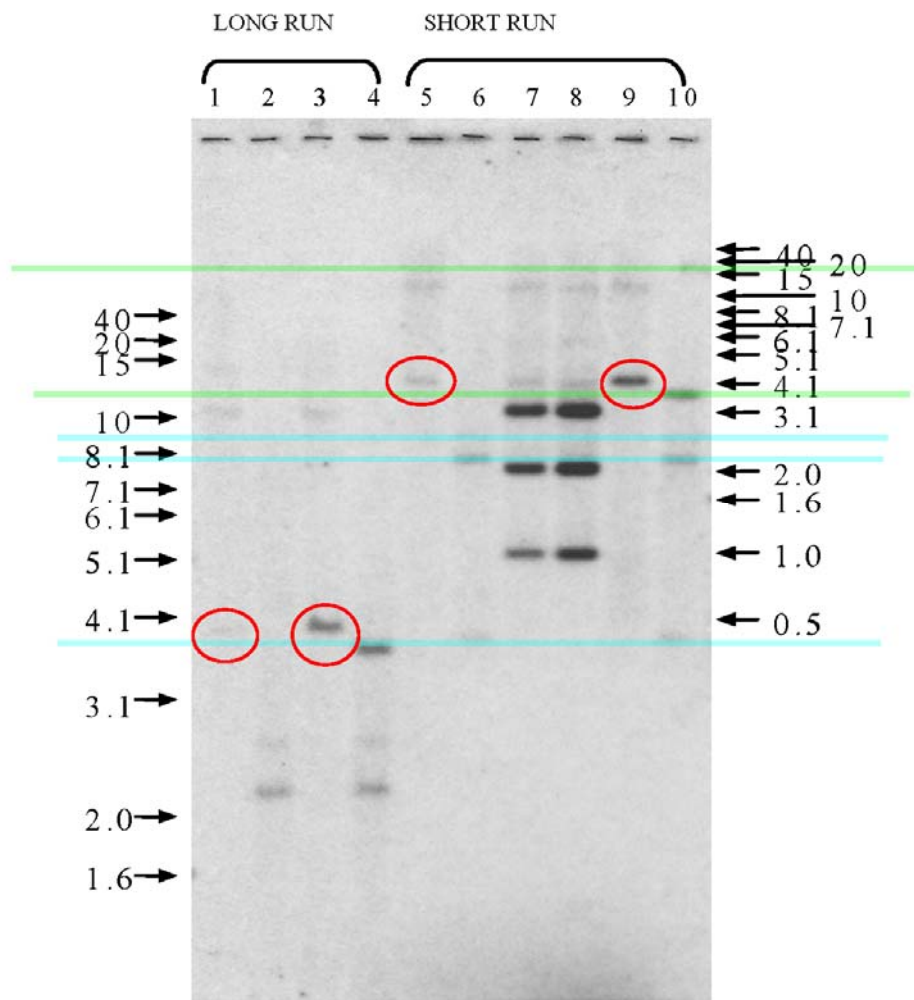


Figure 2

Reproduction of Figure 19 from Application A549 (Monsanto Australia Limited 2004, p. 33). Green horizontal lines (top 2) are bands unique to Lysine maize LY038 (Xho I and Xba I). Cyan horizontal lines (bottom 3) are bands seen in both Lysine maize LY038 (Xho I and Xba I) and LY038(-) (Xho I and Xba I). Red circles are bands that may be in common in both LY038(-) (Spe I) and Lysine maize LY038 (Spe I), and in LY038(-) (Spe I) and Lysine maize LY038 (Spe I).

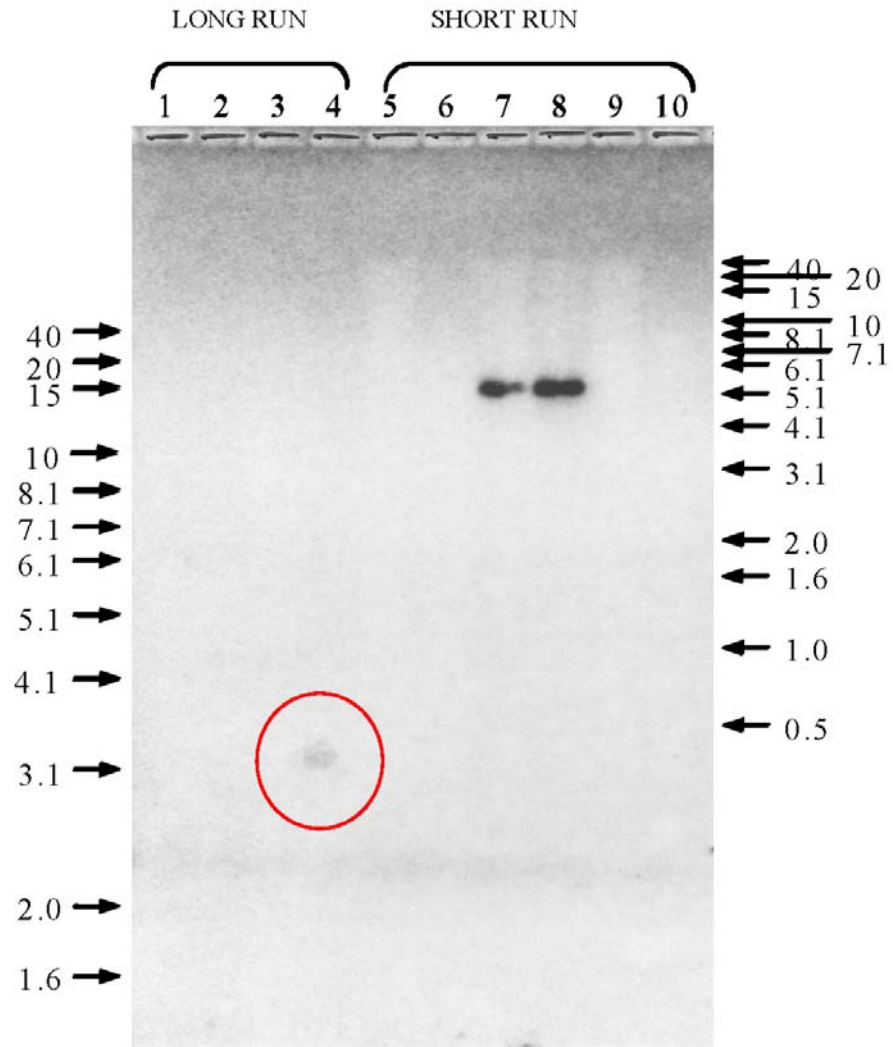


Figure 3

Reproduction of Figure 21 from Application A549 (Monsanto Australia Limited 2004, p. 46). Red circle is band that may indicate hybridization between plasmid backbone probes and LY038 genome.

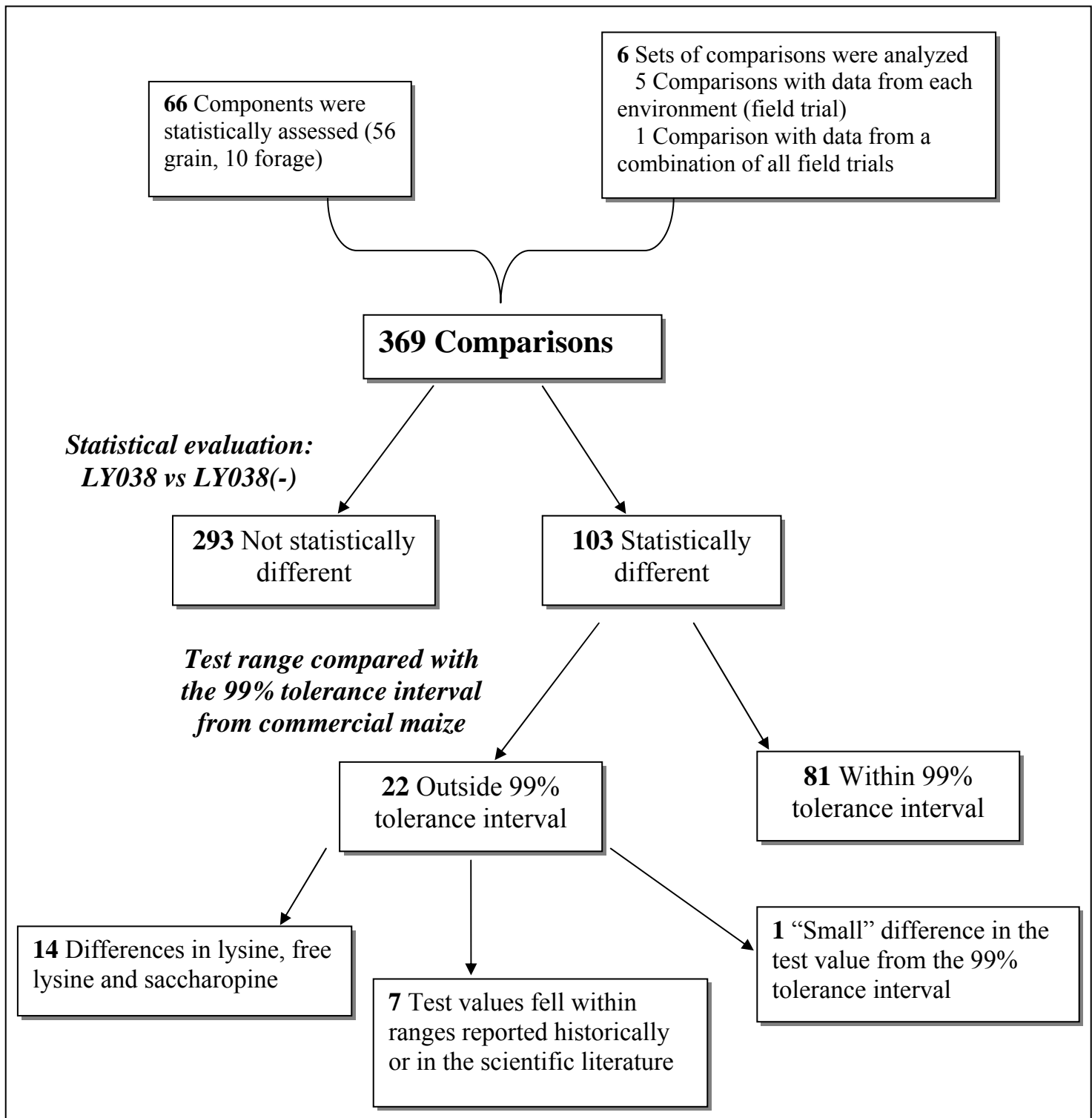


Figure 4

Statistical assessment of the compositional data (MSL-19172).

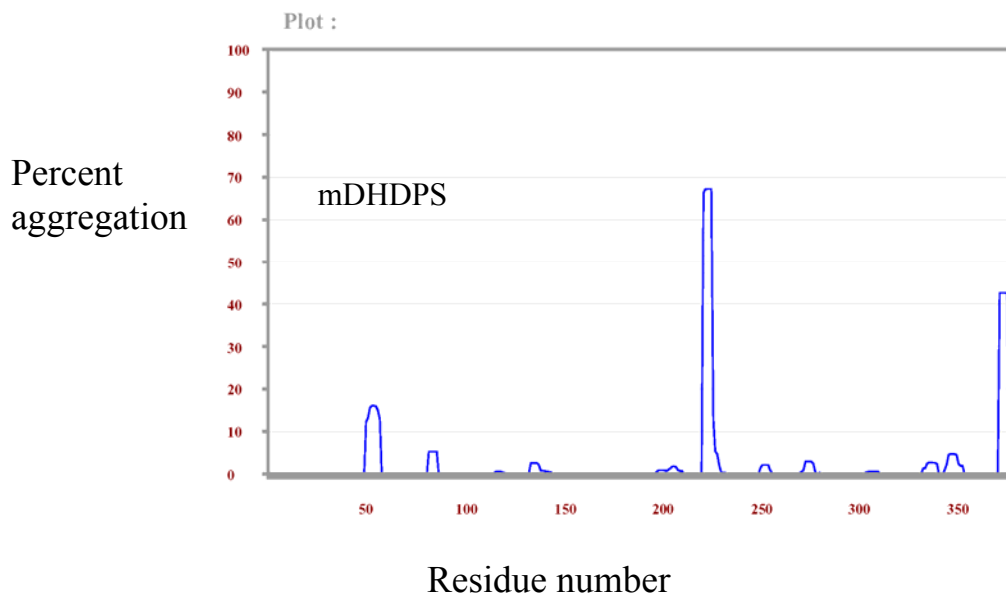
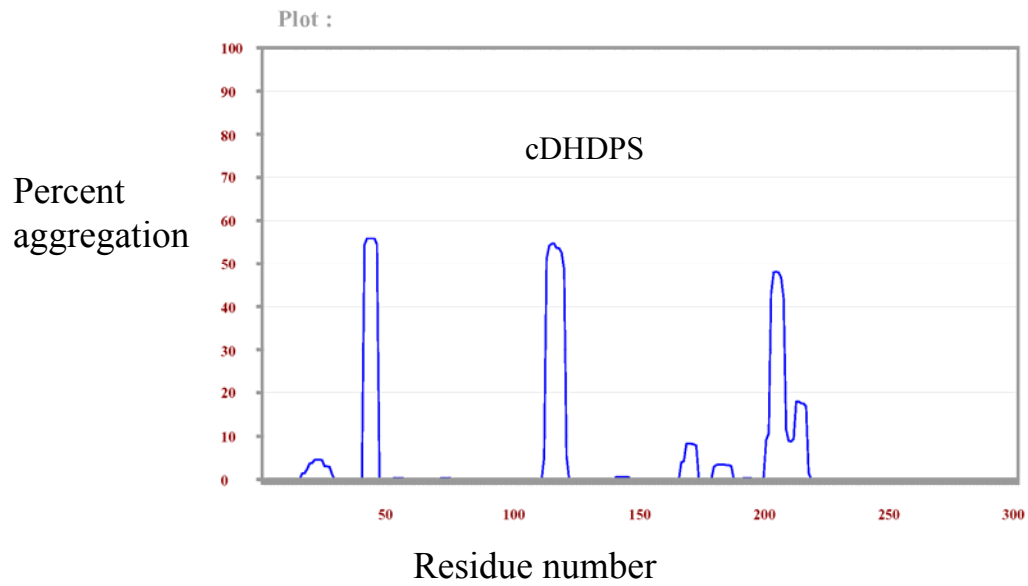


Figure 5
TANGO graphical predictions of aggregation potential and regions of greatest aggregation potential for cDHDPS and mDHDPS.

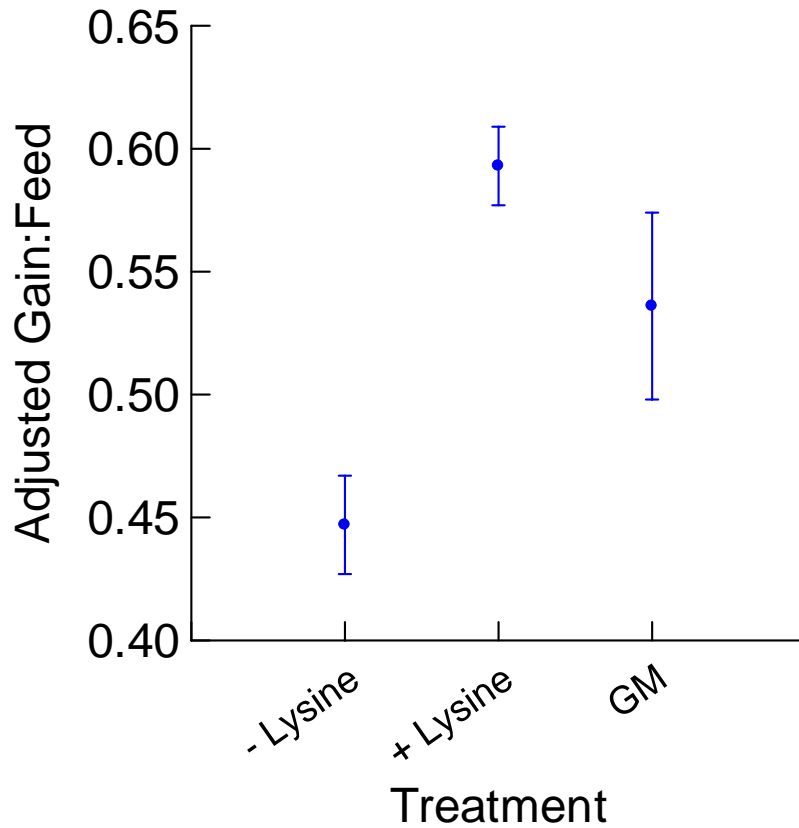


Figure 6

Mean and 95% confidence intervals of Adjusted Gain:Feed for broilers fed different treatments from day 0 – 21. —Lysine (all non-GM maize types without lysine added; n=60), + Lysine (all non-GM maize types with lysine added; n=60) and GM (LY038 and LY038 x MON810; n=20). Data is extracted from the original report and shows a summarized excerpt of Graph G1 in Appendix III.