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Submission on the DAR for **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

Submitter: Centre for Integrated Research in Biosafety
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Corrections to submission

The following errors were discovered after submission. They are listed here to avoid any confusion.

Quote on p. 36 and footnote 11 should refer to
http://www.aphis.usda.gov/brs/aphisdocs/04_22901p.pdf p. 206.

p. 52 The sentence “Inbred D, which is not in the parentage of LY038 (Figure 2), is the closest relative of LY038(-)." This sentence should have been deleted during the final editing stage. It refers to a figure that is no longer in the submission.

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Non-technical summary of key points in this submission

According to the Authority's Draft Assessment Report (section 5.4.2.1), "LY038 corn must be shown to be as safe as other varieties of corn currently available if it is to be approved by FSANZ". Only if the people of New Zealand and Australia can completely substitute LY038, or its descendant hybrids, for conventional corn, and only if they can prepare and eat it the same way and with exactly the same possible consequences as consuming conventional corn, has the Authority met its burden to responsibly recommend that the Food Code be amended.

FSANZ does not have the evidence to declare that LY038 is as safe as other corn when consumed in the same manner. There is strong evidence to suggest that LY038 will produce a spectrum of food hazards significantly different from cooked or processed conventional corn because LY038 has extremely high concentrations of the amino acid lysine and its derivatives saccharopine, α-aminoadipic acid, pipecolic acid and cadaverine. The precise nature of these hazards cannot be adequately predicted from analysis of raw or cooked conventional corn, or raw LY038 corn.

1. Scientific studies on LY038 do not prove it to be as safe as conventional corn

FSANZ spokesperson Lydia Buchtmann was reported as saying to the *West Australian* on 10 April 2006 that "FSANZ used product data from GM companies and compared it with data about conventionally grown food of the same type in deciding to approve products", a process that would be consistent with international standards of review. That standard was not demonstrated in the Draft Assessment Report on A549. In particular, the control did not meet this test in the following respects:

a. The molecular and compositional studies seeking to establish equivalence between LY038 and conventional counterparts remains outside of Codex Alimentarius guidelines[1] and FSANZ policy. LY038 was compared to LY038(-), a sibling of the modified corn line and itself a product of gene technology. In the Authority's own words, "[t]he Applicant has provided information comparing LY038 corn to a closely related control corn crop, LY038(-), both grown in the same location" (DAR p. 11). *LY038(-) is not a conventional food*.

b. The high level of lysine in LY038 corn is dismissed as a dietary risk to humans by saying that "when compared to lysine from other dietary sources this is not a large amount of lysine" (DAR, p. 31 and p. 65). The comparison in this case was to eggs, red meat, chicken, fish, lentils, rolled oats and broccoli, none of which are conventional foods *of the same type as corn*.

c. High levels of the lysine catabolite saccharopine were dismissed as a food hazard by

[1] When referring to the Codex Alimentarius Commission (CAC), we make specific reference to the standards CAC/GL 44-2003 and CAC/GL 45-2003 found at http://www.codexalimentarius.net/web/standard_list.do?lang=en. Access date 26 April 2006.

saying that "[t]he levels of saccharopine found in LY038 corn grain (499 – 818 μg/g dwt, mean 650 μg/g) are substantially higher than those found in broccoli or cauliflower, but similar to the level in button mushrooms" (DAR, p. 48). Button mushrooms are not a conventional food *of the same type as corn*.

d. High levels of the lysine catabolite α-aminoadipic acid, which has a known neurotoxic activity (Rozan et al., 2001), were dismissed as a human food hazard because '[c]ompared to the levels found in other common plant foods, [e.g. lentils, mushrooms, cauliflower, green beans and broccoli] this level is not a cause for concern" (DAR, p. 48). These plants are not a conventional food *of the same type as corn*.

The foods used as a comparison to LY038 differ from corn in the varieties of ways they are prepared, the types of processed foods in which they are found, and in the quantities in which they are consumed. Kiwis and Aussies eat corn chips, but probably do not eat mushroom chips.

2. LY038 has a substantially different potential to create food hazards during cooking

LY038 has high concentrations of compounds that are known to produce food hazards when heated with the sugars found in corn. The modification results in highly elevated concentrations of lysine (total), free lysine (not in protein), saccharopine, α-aminoadipic acid, cadaverine and pipecolic acid, all of which may be converted into advanced glycoxidation endproducts (AGEs) during cooking and processing.

AGEs are strongly implicated in causing a variety of dietary-related diseases including diabetes and Alzheimer's and their sequela (Goldberg et al., 2004, Peppa et al., 2003a, Peppa et al., 2003b, Vlassara et al., 2002), as well as cancer (Heijst et al., 2005). AGE content in food increases with cooking and food processing temperatures and pressures (Elliott, in press, Goldberg et al., 2004).

Compound	Concentration in LY038	Potential Hazard
Lysine	50% higher	AGEs
Free Lysine	50 times higher	AGEs
Saccharopine	110 times higher	AGEs
α-aminoadipic acid	at least 10 times higher	AGEs, neurotoxic
Cadaverine	unknown but expected to be higher	AGEs, accentuates reactions to histamine, evidence of further toxic properties
Pipecolic acid	≥100% higher*	AGEs, chronic hepatic encephalopathy

*Applicant only reports L-pipecolic acid levels. Because D-pipecolic acid can be created from L-pipecolic acid by conversion of either pipecolic acid or lysine to the D-isoform during cooking or in the gut by bacteria, the Authority has likely underestimated pipecolic acid exposure levels derived from high lysine corn or produced by gut bacteria receiving higher levels of dietary lysine.

In their reply to our original submission, the Authority has confirmed that it believes a higher than normal standard of review may be warranted for high-lysine corn. "In cases where the

composition of food has been significantly changed, *as is the case with high-lysine corn,* feeding studies with suitable livestock species may be useful to confirm the wholesomeness of the food" [emphasis ours]. Only feeding studies, using whole plant material in food that has been cooked and processed in ways that humans would consume it, can provide the proper basis for a safety review. No such studies were provided for public review in A549, and from the DAR we have no reason to suspect that such studies were ever provided to the Authority. *We are particularly concerned that the Authority sight, or provide the people of Australia and New Zealand with, reliable data demonstrating that processing and cooking temperatures normal to products that could contain this corn are as safe as products that do contain conventional corn*.

3. Hybrids with LY038 could create significant additional food hazards

The Applicant has assured the Authority that corn derived from LY038 and hybrids will have total lysine in the range of 3500 to 5300 ppm, and free lysine in the range of 1000 to 2500 ppm. However, it is known that research hybrids with parents similar or identical to LY038 could have much higher levels of lysine and free lysine. Free lysine and lysine catabolites were higher in crosses with other GM varieties of corn (Monsanto study published under Huang et al., 2005). The Applicant already possesses hybrid lines of corn with total lysine levels reaching 6160 ppm and free lysine levels reaching 2908 ppm, but apparently did not include that fact in the application.

The Authority has argued that it also cannot restrict its amendment of the Food Code such that future hybrids of LY038 are not automatically approved. This is of concern because LY038 could be bred, on purpose or by accident, with other varieties developed by conventional breeding that might generate substantially increased lysine, free lysine and lysine catabolite levels. If the Authority's recommendation is approved, then it will be authorizing these uncharacterized hybrid varieties to enter the human food supply without further safety review. As we have repeatedly argued in this and a previous submission, the Authority would be making an extrapolation of safety that goes well beyond the scope of the existing scientific data.

Automatic approval of hybrids formed between an approved GM event and a conventional variety, or between two separately approved GM events, leaves the Authority in the potential position of mechanically approving a hybrid high lysine variety with significantly higher levels of lysine. The Applicant has already reported the existence of additional high lysine varieties produced using gene technology. Those varieties achieve high lysine levels through a different biochemical mechanism that works synergistically with the modification reported in LY038 (Huang et al., 2005). The synergistic effect reveals that, in "stacked" varieties (hybrids with both modifications) the levels of free lysine and lysine derivatives are higher than would be expected from an analysis of the modifications kept separate in different varieties. The Authority should indicate what levels of lysine and lysine catabolites it would consider to be potentially dangerous. If the Authority cannot restrict approval to the line described in A549, or provide reason to believe that future hybrids used in human food will not achieve dangerous levels of lysine and lysine catabolities, then it should not approve the LY038 event.

Should the Authority recommend a change in the Australia New Zealand Food Code to allow LY038 and its derivatives, it does so in the knowledge that total free lysine and lysine catabolite levels could reach significantly higher levels in LY038 hybrid corn varieties that do not require a safety assessment.

4. Recombinant protein has no history of safe use

The Authority should have undertaken work aimed at establishing that cDHDPS has a history of safe use by humans as food. However, the Authority has not reviewed data using whole plant derived material (grains) in feeding studies that demonstrate that the primary recombinant protein in LY038, cDHDPS, and its *in planta* produced derivatives, can be consumed safely by humans after normal cooking. Moreover, structural comparisons between cDHDPS (recombinant protein) with the natural corn DHDPS (mDHDPS) demonstrate non-equivalence (Blickling et al., 1997). Therefore, the safety of cDHDPS in cooked human food cannot be extrapolated from the historical presence of mDHDPS in cooked human food. In addition, there is no evidence that humans have been exposed to cDHDPS from natural sources at anywhere near the concentrations that they will be exposed to cDHDPS through eating LY038 corn. We estimate daily human exposure to cDHDPS from natural sources to be between 30 billion-4 trillion *times* less than exposure through LY038 corn (Table 6).

5. LY038 has been tested as an animal feed, not a human food

LY038 is the first genetically modified crop plant substantially different in its nutritional profile to be considered for approval as a human food. In this way, A549 is an application that differs from all previous applications for amendment of the Food Code. The novelty of this product requires, in our opinion, adherence without exception to the highest standard of review and international consensus standards for review, such as described by the international bodies the Codex Alimentarius Commission (CAC), the UN Food and Agriculture Organisation (UN FAO), and the World Health Organisation (WHO).

Despite its statement to the contrary (DAR section 5.4.2.1, p. 13), we believe that the evidence reviewed by FSANZ falls short of the evidence necessary to assure it is as safe as conventional corn. This is no surprise because the evidence provided was produced to assess LY038 only for use as animal feed. The key difference between the use of corn as an animal feed and a human food is cooking and processing, and the Authority has made no attempt to assess food hazards resulting from cooking or processing of LY038.

Frequent reference by FSANZ to the Applicant's "intent", and to future market forces, to limit incorporation of LY038 into human food implies an added safety margin that is both inappropriate (because amendment to the Food Code does not bind the Applicant to keep LY038 out of the human food supply, restrict the foods that LY038 is used in, or to minimize co-mingling with varieties that are used in human food) and is no reason for allowing its tests as an animal product to substitute for proper human food safety tests. Examples of such references in the DAR are reproduced in the table below.

"Furthermore, little LY038 will be entering the food supply, mostly in the form of processed products (e.g. corn syrup) that contain negligible amounts of protein"	DAR p. 11

"Although LY038 will be grown as a high value animal feed, a small percentage of this corn may enter the food supply"	DAR p. 20
"because LY038 corn is not intended for food, human consumption is expected to be extremely low"	DAR p. 23
"Further, it is expected that the amount of LY038 grain entering the food supply will be small"	DAR p. 31
"It is less likely that food industry would pay premium price for high-lysine corn and therefore likely that the levels of high-lysine corn entering the food supply would be small"	DAR p. 72

6. The Authority has accepted a standard of evidence of safety that is below what it could request under international guidelines

International bodies have set higher standards for the description and testing of genetically modified food organisms, such as LY038, that are significantly different from their conventional counterparts. According to CAC, the Authority could ask for:

a. feeding studies using LY038 grains cooked and processed in ways that humans prepare corn for food to identify food hazards that derive from, for example, unusually high concentrations of AGEs. "The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered."

b. feeding studies using cooked and processed LY038 grains to determine the potential for cDHDPS to form toxic aggregates or sugar-protein derived allergens (another AGE product). "The absolute exposure to the newly expressed protein [cDHDPS] and the effects of relevant food processing will contribute toward an overall conclusion about the potential for human health risk."

c. a compositional analysis using a comparator that was "the near isogenic parental line", and only if this were not feasible should the Authority consider another line that was "as close as possible". In this particular case, the Authority does not have to accept the use of LY038(-) as a control because the non-GM parental line, H99, is 65.6% identical to LY038. We have seen no evidence to prove that LY038(-), the GM sibling line, is above 50% related, the average relatedness of siblings.

Despite compelling scientific evidence that food hazards will form when corn derived from LY038 is cooked, and that the absolute exposure to cDHDPS will be astronomically higher than from natural sources, the Authority has not required studies that would be necessary to detect the presence of hazards specific to the use of LY038 as a human food. The Authority should explain why it believes that it is satisfactory to allow high lysine corn into the food supply following a safety review whose standards, in important respects, is frequently below what is allowed and recommended by international intergovernmental food safety agencies when this is acknowledged to be an important precedent.

7. A recommendation to amend the Food Code does not follow from a case-by-case assessment

The Authority has expressed its commitment to case-by-case assessment. "The safety of GM foods cannot be assessed as a single class because the safety concerns depend on the type of food and the nature of the genetic modification. For this reason, safety assessments are performed on the foods derived from *individual types of GM plants or animals*"[2]. The Draft Assessment does not, however, adhere to its case-by-case assessment policy because the Authority is drawing general safety conclusions from experience with different types of modified corn. For example, the Authority's statement that—

> "[t]o date, all approved GM plants with modified agronomic production traits have been shown to be compositionally equivalent to their conventional counterparts. Feeding studies with feeds derived from the approved GM plants have shown equivalent animal performance to that observed with the non-GM feed. Thus the evidence to date is that for GM varieties shown to be compositionally equivalent to conventional varieties, feeding studies with target livestock species will add little to a safety assessment and generally are not warranted" (DAR p. 49).

is not specific to LY038, *its use in human food*, or the potential hazards that have been identified for LY038. Such use of evidence is outside the case-by-case assessment framework.

In contrast, our submission is composed of an in depth examination of the scientific studies submitted to the Authority by the Applicant. This examination is supported by an analysis of up-to-date, peer-reviewed, scientific literature. This literature is specific to hazard identification or evaluation, consistent with CAC and OECD[3] recommendations.

The Authority (FSANZ) has committed itself to making assessments "based on risk analysis using the best available scientific evidence"[4]. *Our submission is based on the best available scientific evidence.* It has been updated with relevant references as recent as early 2006. Thus, we believe that to be consistent in the 'case-by-case' approach to assessment, and to be consistent with international recommendations for hazard identification, the Authority must refrain from substituting unsupported speculation—such as "expected to be", "not expected", "considered to be" or "not considered to be"—for hard scientific data, and either dismiss, or justify its use of, data that are not specifically relevant to High-Lysine Maize LY038, in reply to our specific analysis of LY038.

8. Conclusions

We believe that too much legitimate scientific uncertainty exists after consideration of the scientific studies submitted in support of A549 for the Authority to assert that LY038 and any hybrids derived from it are as safe as food derived from conventional corn. There is no case

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[2] ANZFA Occasional Paper Series No. 1 GM foods and the consumer (2000).

[3] When referring to the OECD, we make specific reference to the Consensus Document on Compositional Considerations for New Varieties of Maize (*Zea Mays*): Key Food and Feed Nutrients, Anti-Nutrients and Secondary Plant Metabolites (ENV/JM/MONO(2002)25) from OECD found at http://www.oecd.org/document/9/0,2340,en_2649_34385_1812041_1_1_1_1,00.html (access date 2 May 2006).

[4] FSANZ (2004). Initial Assessment Report: Application A549 Food Derived from High Lysine Corn LY038, p. 8-9.

made for a benefit to Australians or New Zealanders to have LY038 in their food. There is considerable evidence of probable harm in comparison to conventional corn.

In our view, the Authority is making a recommendation that is also inconsistent with Codex Alimentarius. At the very least, the Authority should commission the following:

- a compositional study using H99 as the control in five sites over at least two years because H99 is the closest relative of LY038 and is the non-GM parental;
- a compositional study describing the compounds formed during heating and processing of LY038 corn material as it would be in human foods—using the parental varieties as controls;
- an animal feeding study using whole food derived from LY038 corn heated and processed as per normal use in human food—using the parental varieties as controls; and
- human exposure studies (should the previous two studies not reveal clear hazards) that measure the effects of using whole food prepared from LY038 corn, pipecolic acid levels contributed from gut bacteria, and the potential for an allergic response to LY038 following inhalation of LY038 flour.

The Authority, if it ultimately recommends an amendment to the Food Code, should restrict that approval to the specific line evaluated in A549, and ensure that the approval cannot be extended to hybrids. The authority should also impose an actively managed post-marketing monitoring programme.

Guide to this submission

Overview

This submission from the Centre for Integrated Research in Biosafety (INBI) is meant to support Food Standards Australia/New Zealand's preparation of a Final Assessment on application A549.[5] INBI is dedicated to development for the public good of all responsible biotechnologies. We are an assemblage of well-recognized researchers with independent credentials in the area of biotechnology and its social impact.

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.[6] 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."[7]

Our submission is to form part of the consultation process of the FSANZ Draft Assessment Report and provisional recommendation that the Food Code be amended to include LY038. This submission follows from our February 2005 submission at the previous stage of consultation (made under our previous name, the New Zealand Institute of Gene Ecology), following the release of the Initial Assessment Report (IAR). We begin with introductory material describing who we are and why we are involved. The major content of our submission is organized into four main parts. In Part One, we address Attachment 4 of the Draft Assessment Report (DAR) prepared by FSANZ, which is the response to our February 2005 submission (NZIGE submission). In this section, we set out the deficiencies in the FSANZ response and make recommendations for rectifying those deficiencies.

[5] Our previous submission was under the name the New Zealand Institute of Gene Ecology.
[6] FSANZ (2004). Initial Assessment Report: Application A549 Food Derived from High-lysine Corn LY038, p. 6.
[7] Ibid, p. 9.

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 Authority should seek further information before it advises the Ministerial Council that food derived from LY038 and derivatives is as safe as food derived from conventional counterparts.

In Part Three, we comment upon the Impact Analysis and the conclusions derived from it. We assess the costs and benefits listed and propose further costs and benefits of the options under consideration.

In Part Four, we note several issues related to FSANZ's consultation process that require clarification, including the treatment of our submission to the A549 Initial Assessment Report.

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Common abbreviations used:
ADF: acid detergent fibre
AGE: advanced glycoxidation endproduct
CAC: Codex Alimentarius Commission
cDHDPs: dihydrodipicolinate synthase derived from *Corynebacterium glutamicum*
CJD: Creutzfeldt-Jakob disease
DAR: Draft Assessment Report
g: grams
GRAS: generally regarded as safe
IAR: Initial Assessment Report
mg: milligrams (10^{-3}g)
μg: micrograms (10^{-6}g)
NDF: neutral detergent fibre
NZIGE Submission: 25 February submission by the New Zealand Institute of Gene Ecology to FSANZ on the IAR
OECD: Organisation of Economic Cooperation and Development
ppm: parts per million
TDF: total dietary fibre
UN FAO: United Nations Food and Agriculture Organisation
WHO: World Health Organisation

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 Centre for Integrated Research in Biosafety (INBI), a research organization (www.inbi.canterbury.ac.nz). INBI 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. Our submission is informed by our 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" (DAR p. 6). It also addresses the consistency of the DAR 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" (DAR p. 3).

I.4 We have done our best to evaluate the scientific documents supplied by the Applicant in support of the application.

Part One: Evaluation of Attachment 4, DAR

"At Initial Assessment, the New Zealand Institute of Gene Ecology (NZIGE) reviewed all the data submitted to FSANZ by Monsanto in support of this application and compiled a detailed submission, outlining a number of areas where it believes there are deficiencies in the safety data."[8] In fact, INBI reviewed all *publicly available* data submitted to FSANZ by Monsanto. FSANZ makes reference to several studies, including a rat study, two bioinformatics studies and a compositional study, that were not made available to us during the last round of consultation.

In its response to the recommendations made by INBI, we believe that FSANZ has overlooked the extensive discussions underlying the recommendations. In many places, the FSANZ focus on the recommendation has missed important areas of scientific uncertainty. Here we will address the response made to the original submission, indicating in brackets the index numbers corresponding to the text from the original submission that explains the recommendation. The following sections are ordered as per the FSANZ response to the recommendations in our original submission (25 February 2005).

FSANZ response to recommendation 1: Characterisation of novel protein, intended and unintended effects

> "Although general profiling techniques such as proteome analysis can be valuable research tools, a major challenge is in determining whether observed differences between two plants are distinguishable from natural variation, and if so, their relevance to food safety. For such techniques to be of use for the purpose of regulation they must be validated and the baseline range of natural variation clearly established (ILSI, 2004)."
>
> FSANZ has evaluated the molecular analysis of the inserted DNA in the genome of LY038 corn, which shows that only one copy of the gene encoding cDHDPS is present. No other novel proteins are produced in LY038. A full characterisation of this protein was done as part of the safety assessment of LY038 and is described in Section 4 of Attachment 2 of the Draft Safety Assessment Report. The data provided was comprehensive and included N-terminal sequencing, molecular weight determination, immunoreactivity, glycosylation analysis, peptide mass fingerprinting and matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry. Further information such as suggested in this recommendation would not add to characterisation of cDHDPS and therefore has not been requested by FSANZ."

INBI: (1) Profiling techniques can be used in two different ways, only one of which is addressed in this response. (2) Our recommendation was not just for untargeted profiling, but for specific data on the full range of cDHDPS isomers produced in transgenic plants. (3) The extensive characterizations described above could not provide insight into isomers that the Applicant failed to identify in the first place.

(1) First, as indicated, an unintended effect may be revealed through a statistically significant change in the concentration of a harmful metabolite, for example, protein, class of RNA or

[8] DAR, p. 63.

other molecule. To establish statistical significance may require the Applicant to generate good baseline data and the Industry to invest in proper validation techniques. It is not the public's responsibility to ensure that these verifications are completed for the Applicant.

Second, as revealed in the full quote from ILSI, profiling techniques can be used to identify unintended changes that may be harmful to consumers even in the absence of data that indicates that gross changes are statistically significant. "Predictable and unpredictable unintended effects may or may not prove to have relevance in terms of product safety, but must be taken into account when assessing risk" (Cellini et al., 2004). Codex Alimentarius says "[m]olecular biological and biochemical techniques can also be used to analyse potential changes at the level of gene transcription and message translation that could lead to unintended effects" (p. 10). Even the Applicant has concluded that "this method [2D gel electrophoresis] could be used to interrogate proteome alterations such as a novel protein, fusion protein, or any other change that affects molecular mass, isoelectric point, and/or quantity of a protein" (Monsanto study published under Ruebelt et al., 2006a). The Applicant has published demonstrations of the application using transgenic *Arabidopsis* plants: "This study demonstrated that 2DE [2D gel electrophoresis] *can be utilized to reliably analyze the seed proteome* of transgenic *A. thaliana*" (emphasis ours) (Ruebelt et al., 2006b).

While the profiling techniques may not be of the sophistication necessary to rule in or out harm by themselves (Ruebelt et al., 2006b), the Applicant should be responsible for pursuing unintended effects by whatever techniques are available to a point that would reasonably satisfy a regulator using international standards for safety assessment that the change was not meaningful.

For example, the gross change in protein concentrations between healthy bovine and those suffering from Mad Cow's Disease may not be statistically significantly different, but the change in the protein profile of bovine suffering from the disease would still be a matter of importance for assessing the beef as safe for humans to eat. Profiling techniques are capable of being used in this way (Riemer et al., 2004). Any differences detected can then be investigated. So while profiling does not produce final evidence of harm, failure to detect differences is an indicator of safety.

(2) The Applicant's case for safety requires that *in planta* produced cDHDPS is equivalent to the material that the Applicant isolated from other, surrogate, sources (e.g. *Escherichia coli*) because many of the Applicant's subsequent analyses use protein isolated only from surrogate sources. We believe that FSANZ has an obligation to the public of Australia and New Zealand to verify scientifically that *in planta* produced cDHDPS is equivalent in all ways.

(3) The two methods that the Applicant used to isolate cDHDPS from transgenic plants were both potentially compromised by post-translational modifications unique to the production of the protein in plants (5.3.15).

> **"[I]t is important to note that there are fundamental differences between the identification of a protein and the analysis of its post-translational modifications. Minimal sequence information will suffice to ensure unambiguous protein identification but practically full sequence coverage must be obtained in order not to miss the (few) modified amino acids" (Küster et al., 2001).**

The only scientifically sound way to confirm that there are no unique isomers of *in planta* produced cDHDPS is to use comparative 2D gel electrophoresis and MS.

This fact was made most dramatically by authors of studies applying different techniques to catalogue the glycoforms of the human Prp^c protein (the benign form of the human prion protein). A recent study found that many isomers, some with significant N-terminal truncations that they argue are not simple autolysis products (as the Applicant has argued for some rogue bands that appear on Western blots in this application), would not be detected by 1D approaches used in the studies provided to the Authority. "It is probable that, without resorting to 2-D map analysis, as in the present case, only a few spots of PrP might have been identified, but it is highly doubtful that such mono-dimensional techniques, even when coupled to the most sofisticated (sic) MS instrumentation, would have revealed the amazing structural complexity of PrP proteins from human tissues, where >60 different spots (vs. 3-4 by SDS PAGE) could be visualized" (Castagna et al., 2002).

The Applicant's use of purified protein followed by SDS PAGE and MALDI-TOF to verify the identity of the protein is not equivalent to a conclusion that all potential isoforms of the protein produced *in planta* were detected. The combination of techniques used verify protein identity, but are not verified as used as a pathway to a comprehensive isoform discovery (Küster et al., 2001). The key data equating the *E. coli* and *in plant*a-produced recombinant cDHDPS (Figure 30, p. 50, of application) has several flaws for drawing the conclusion of identity.

- The antiserum used in the Western blot lacks sufficient description. Post-translational modifications vary by species, tissue and time of development, and important epitopes can be masked by post translational modifications (Küster et al., 2001). Thus, the Authority cannot know that the antisera includes antibodies that would detect minority glycoforms, or other isoforms, unless it can verify that the goat anti-cDHDPS antisera was raised to protein isolated from the plant.
- The protein loaded onto the SDS gel was purified using a combination of anion exchange and hydrophobic interaction chromatography, both of which can be affected by post translational modifications (and thus bias against detecting some post translational modifications) (Küster et al., 2001). Thus the Authority cannot know that all minority isoforms were retained in the protein fractions loaded onto the gel.
- The analysis from which the Authority is working does not exclude either co-migration of isoforms with the detected form, or other bands on the gel but below the detection level. "1 pmol of a glycoprotein on a gel will represent a composite of many glycoforms present only in the low fmol range" (Küster et al., 2001). The Authority should know and report the detection level of this particular Western.
- SDS PAGE does not detect modifications that contribute less than 1 kDa to molecular weight. Many types of modifications involve molecules less than 1 kDa.

We also note that there are difficulties in interpreting 2D gels (Küster et al., 2001), but the difficulties are in distinguishing between kinds of post translational modifications rather than a bias against detecting variant isoforms. "Although this global view on protein glycosylation does not allow the detailed description of all aspects of glycoform heterogeneity, it is a good first round analysis in order to assess the overall complexity of the sample and thereby assists in deciding whether or not to investigate glycan structure in more detail" (Küster et al., 2001).

Thus, 2D gels are an essential complement to the Western analysis provided.

While FSANZ may be choosing to conclude that all novel proteins not described by the Applicant are not required to be, it is not scientifically accurate for FSANZ to conclude from the data provided by the Applicant that "[n]o other novel proteins are produced in LY038." Further, we are not able to determine how FSANZ can know that the outcome of scientific experiments looking for unanticipated changes "would not add to characterisation of cDHDPS" when those experiments have never been done. A sound scientific assertion of safety that FSANZ draws from the Applicant's data requires, in our opinion, properly conducted profiling experiments that will detect unintended changes followed by whatever techniques the Applicant can use to evaluate those changes.

Please see Part Two, "Characterisation of the novel protein", for further discussion and recommendations.

FSANZ response to recommendation 2: Toxicity, carcinogenicity and whole food feeding studies

> "It is unclear whether this recommendation refers to feeding studies with the purified protein or with the whole food. Long term feeding studies in animals are of limited value for determining the safety of whole foods, including GM food. The safety assessment of a GM food relies largely on a comparison with its conventional counterpart. This is discussed further under recommendation 12.
>
> The biochemical components identified in LY038 corn are the normal constituents of conventional corn with the exception of the cDHDPS protein. This protein is structurally and functionally related to an endogenous DHDPS protein. As with any whole food, incorporation of LY038 corn into the diet of animals at high levels and over long periods of time is likely to cause physiological and/or biochemical changes due to nutrient imbalances, rather than any specific toxicity. In terms of the contribution of diet to cancer risk, over the long term a balanced diet of nutritious foods is probably more important to health that the level of intake of any one food.
>
> There is no scientific justification for FSANZ to request lifetime carcinogenicity studies with LY038 corn."

INBI: (1) Feeding studies are valuable studies to reveal unanticipated harms. (2) It is both wrong and misleading to imply that *in planta*-produced cDHDPS is structurally equivalent to the plant form of DHDPS just because they share an ancient evolutionary relationship (1.1.3). (3) Products unique to LY038 or at unique concentrations in LY038 that are produced during cooking (2.1) may lead to cancer. In the absence of a complete profile of novel forms of proteins (see above), RNA and metabolites in LY038, there is no scientific justification to assert that lifetime carcinogenicity studies and aggregation studies would not be valuable. See Part Two of this submission, "Characterisation of novel protein", for additional analysis.

(1) FSANZ assertions of safety should be demonstrably consistent with the results of animal feeding studies using whole cooked and processed food.

(2) The bacterial and plant versions of DHDPS enzymes have an entirely different quaternary structure due to a profound rearrangement of the dimers forming the tetramer (Blickling et al.,

1997).

(3) 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. Moreover, those studies did not use cooked or processed corn. As we have argued, the high levels of lysine, free lysine and lysine catabolites makes LY038 corn a potential source of high AGE content in food. "CML-modified proteins [lysine AGE endproducts] may be involved in the biology of tumors through the activation of transcription factor NF-κ, the up regulation of vascular endothelial growth factor (VEGF), vascular cell adhesion molecule 1 (VCAM-1), tissue factor (which could explain the CML positivity of capillaries), and the induction of intracellular reactive oxygen species. In addition, the presence of CML could lead to enhanced cancer progression by inducing DNA damage" (Heijst et al., 2005). Thus, long term carcinogenicity studies are warranted.

See the next response for more information on AGEs.

FSANZ response to recommendation 3: Food Processing, Maillard reaction

> "This recommendation is based on the concern that any increase in lysine in food may lead to adverse effects, for example, by altering the Maillard reaction products when LY038 corn is cooked…However, Maillard reaction products produced during the processing or cooking of LY038 grain should not differ from those produced from conventional corn. With the exception of lysine, the amino acid profile of LY038 grain is comparable to conventional corn.
>
> It is well accepted that any normally healthy compound, when consumed in sufficiently large quantities, may cause health problems, however the levels of lysine in LY038 corn do not warrant the level of concern displayed by the NZIGE. Lysine is an essential amino acid that cannot be produced by humans and therefore must be obtained from the diet. Corn is not traditionally a good source of lysine and even though LY038 contains significantly increased lysine, this is not significant in relation to other dietary sources of lysine. The levels of lysine in some commonly consumed foods are given in the following table [not reproduced here].
>
> It can be seen that LY038 grain has approximately 160 mg / 100 gm more lysine than the control corn grain, however when compared to lysine from other dietary sources this is not a large amount of lysine and does not represent a human health concern."

INBI: (1) AGEs may differ between LY038 and conventional corn after processing or cooking because—as FSANZ, the Applicant and we all agree on—the lysine concentration and other Maillard reactants are significantly higher and at unprecedented concentrations (especially free lysine) in a novel context in this transgenic plant. (2) It is neither true nor in our view responsible to suggest that there is historical evidence to dismiss the production of undesirable AGEs or anti-nutrients that would be specific to lysine at these concentrations in corn with its unique types and concentrations of carbohydrates, in the context of the products that corn is most often cooked with, and in the way that corn may be prepared (2.1.2). On the contrary, there is historical evidence of anti-nutrients formed by the Maillard reaction in corn. (3) LY038 must be found to be as safe as conventional corn, not conventional red meat, fish, cheese, chicken, eggs, broccoli, lentils and rolled oats as suggested by FSANZ when drawing comparisons between total lysine in these foods and LY038 corn and inferring the importance

of dietary free lysine derived from cooked LY038 products. LY038 is not substantially equivalent to these foods, conventional or otherwise. (4) FSANZ cannot, in our view, meet its 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"[9], and be consistent with Codex Alimentarius, while allowing a change in the Food Code that would permit the substitution of low AGE content (conventional) vegetables with genetically modified crop plants that could produce higher AGE content foods, considering the importance of AGE-related diseases to Western society.

Please see the "Food Processing" section in Part Two for details and recommendations.

FSANZ response to recommendation 4: Characterisation of novel protein, aggregation

> "This concern has been raised by the NZIGE because amyloid fibrils are involved in a variety of medical conditions such as Alzheimer's and Parkinson's diseases. However, these fibril aggregates are produced from endogenous proteins that have sustained mutations or have been misfolded, rather than from the consumption of particular proteins.
>
> The ability to form fibrils is not limited to those proteins involved in amyloidoses, it appears that any polypeptide can be induced to form fibrils under appropriate conditions in vitro (Chiti et al., 2000; Ellis and Pinheiro, 2002; Bucciantini et al., 2002). There is also some evidence that protein aggregates are inherently cytotoxic (Bucciantini et al., 2002). Therefore testing cDHDPS to determine if it forms cytotoxic fibrils would not provide useful information for a safety assessment of LY038 corn.
>
> The cDHDPS protein is no more likely to form amyloid fibrils than any of the naturally occurring proteins in LY038. Even in the event that cDHDPS aggregates form in planta, a series of improbable events would have to occur in order for cDHDPS fibrils to display cytotoxicity in human cells. FSANZ is of the opinion that the studies submitted by the applicant demonstrate the safety of LY038 corn and do not believe that results of such a study as suggested in this recommendation would add to the overall body of information."

INBI: (1) Amyloid fibrils are produced from endogenous proteins, but it is incorrect and misleading to say that they are not spread through the consumption of particular proteins. (2) It is impossible without testing to conclude that cDHDPS, in corn cells and through processing of foods with corn content, is "no more likely to form amyloid fibrils than any of the naturally occurring proteins in LY038". (3) The Authority should explain why it believes that the connection between cDHDPS aggregates and cytotoxicity relies on a series of improbable events, considering that there is published evidence of the cytotoxicity of protein aggregates.

(1) Some aggregates do transfer through food and are infectious. Variant CJD (vCJD) disease in the UK is caused by the consumption of beef derived from diseased animals. Aggregates formed by similar proteins, those that cause CWD (the cervid version of mad cow disease) are stable in soil for at least two years and can be transmitted between animals through contact, through grazing near rotting carcasses of diseased animals or through feces (Miller et al.,

[9] Ibid, pp. 8-9.

2004). These examples directly demonstrate the ability of xeno-aggregates to transfer their effects to endogenous proteins in humans and other mammals.

(2) Our argument is not that cDHDPS aggregates will create prions in human consumers or cause prion-associated diseases in human consumers. Our argument is simply that protein aggregates of many forms, including amyloid fibrils, can be shown in laboratory experiments to be cytotoxic. cDHDPS is not a normal constituent of human food (see Table 6), and certainly not at the concentrations that LY038 and its derivatives may make it. cDHDPS is not the same protein as the endogenous DHDPS and cDHDPS does not normally reside under physiological conditions of the plant chloroplast. Thus, its tendency to form aggregates of potential cytotoxicity cannot be determined by argument or reliance on GRAS.

(3) In our original submission, we provided the Authority with a list of references describing the current scientific view of how proteins form aggregates and how this process was a function of milieu conditions. We also provided the Authority with references (e.g. Bucciantini et al., 2002) demonstrating that proteins derived from natural sources generally regarded as safe can be cytotoxic if allowed to re-fold under different conditions. It is now the burden of the Authority to provide the people of Australia and New Zealand with evidence for their statement that a "series of improbable events would have to occur in order for cDHDPS fibrils to display cytotoxicity in human cells."

We are aware that Monsanto provided the "improbable events" rationale to the Authority and at the Authority's request (as it has for most of the FSANZ responses in Attachment 4, DAR). The original Monsanto advice provided to the Authority reads as follows, with INBI comments in brackets:

"In the event the cDHDPS were to form amyloidal fibrils or aggregates *in planta*, these fibrils or aggregates would have to exit the chloroplast intact and gain access to the cytoplasm of a susceptible cell." **[It is necessary for the Authority or the Applicant to provide evidence that chloroplast proteins are completely destroyed by digestion within the plant or human consumer. We are not aware of such evidence. On the contrary, we are aware of evidence demonstrating that many proteins survive digestion. We are also aware that proteins transmitted through food do gain access to the cytoplasm of a large range of human cells. This has been demonstrated by prions (Soto, 2004), and was the reason we discussed prions in our original submission.]** "Once in the cytoplasm of a susceptible cell, the fibril would then have to either exert cytotoxicity directly, or act as the nucleus for the aggregation/fibril formation of an endogenous protein(s). In the unlikely event that cDHDPS forms *in planta* aggregates…" **[We believe that it is necessary and possible to remove this issue of uncertainty by measuring the propensity of *in planta*-produced cDHDPS to form aggregates and relate that propensity to the conditions of the chloroplast. There is no scientific reason to assume that aggregation is unlikely.]** "*…a series of improbable, if not physically impossible events would have to occur in order for cDHDPS fibrils to display cytotoxicity*" (Monsanto reply to FSANZ).

Please see Part Two "Characterisation of novel protein, aggregation" for further discussion and recommendations.

FSANZ response to recommendation 5: Molecular characterisation, untranslated RNA

> "The rationale behind this recommendation is presented in the NZIGE submission in Section 1.3. This section presents a summary of the biological properties of RNA that is generally accurate. However, the scientific evidence does not support the theory that RNA molecules in food can be transmitted to mammalian cells and exert effects on endogenous genes.
>
> RNA is rapidly degraded even in intact cells. Following harvest, processing, cooking and digestion, it is unlikely that intact RNA would remain. Even if it did, it is very unlikely that it would enter human cells and be able to exert effects on endogenous genes.
>
> What little is known about transcription levels of genes across entire plant genomes indicates that gene transcription may vary considerably even between closely related plants (Bruce et al., 2001; Guo et al., 2003; Umezawa et al., 2004). This high level of differential expression is thought to be due to a number of factors including environmental conditions and genotype. For this reason, analysis of changes in the transcriptome, while interesting, would not indicate whether these changes are within the range of natural variation nor would it provide any further information on the safety of the food."

INBI: (1) The Authority has avoided the recommendation and replied with no scientific evidence to support their assertion that dsRNA of the type involved in phenomena such as RNAi, PTGS, etc. are so unstable as to not have biological relevance. (2) In fact, evidence to the contrary is rapidly accumulating. (3) In addition to the extensive discussion in our original submission (1.3), and its list of references, we have these comments.

(1) The Authority is requested to note that dsRNA are stable enough in mammalian cells to be routinely used as gene regulators. Up to one third of human genes are regulated by dsRNA (Lewis et al., 2005). It is transmitted through food in other animals, where it survives degradation in bacteria producing the molecule and digestion in the animal gut. The medical literature is already exploring the use of exogenously developed dsRNA as therapeutics (Stevenson, 2004). Delivery mechanism in mammals include through food (Brisibe et al., 2003) and injection (e.g. Lewis et al., 2002). Injection of dsRNA into the tail of a mouse affects gene expression in broadly distributed organs, thus demonstrating its stability and transmissibility inside mammals (e.g. Check, 2004, Zamore and Aronin, 2003).

(2) As of May 2006, it has been demonstrated the dsRNA constructs produced in *E. coli* can effect gene silencing (RNAi) in the gut cells of mice (Xiang et al., in press). Mice were fed *E. coli* expressing dsRNA directed against genes in the intestinal cells. In this study, the bacteria were nonpathogenic but engineered to invade human cells. What the study demonstrates is that dsRNA is stable in *E. coli*, stable in the human stomach under some conditions, survives the intestine and is biologically active in intestinal cells. *The dsRNA was transmitted to the animal through oral ingestion, as if it were food.* The Authority should note that this is what we indicated was possible when we wrote our first submission over a year ago. Although this demonstration was not the same as feeding naked dsRNA, the Authority should also be aware that the dsRNA in LY038 would be protected by the plant cell or cellular debris, or the surface of bacteria, and therefore could very likely survive the stomach. This field of research is so new

that such strong statements of confidence as the Authority has made are inappropriate and lack credibility.

(3) Transcriptional variability in plants is irrelevant to this issue. Variability of transcription is not the cause of novel species of dsRNA; transcription of novel DNA sequences is. It is transcription of novel DNA sequences that were, and may have been, created through insertion of the I-DNA into a traditional food source: corn. What is relevant is whether, as a direct or unanticipated consequence of the modification of corn, a type of dsRNA may emerge that has biological consequences in humans.

The techniques we suggested for discovering novel dsRNAs may not be the only options available to the Applicant. They are, as discussed above, a first step in identifying unanticipated RNA species. If the technique recommended is not used, then the Authority should still sight evidence that all novel RNA species have been identified, characterized and tested for food safety.

Please see Part Two, "Characterization of the genetic modification" for further discussion and recommendations.

FSANZ response to recommendation 6: Glb1 promoter

> "This recommendation has been made due to concerns of the NZIGE that the intact Glb1 promoter sequence and the cordapA gene to which it is attached may be taken up by human cells following ingestion and cause the over-expression of cDHDPS in human cells or deregulate expression of endogenous genes.
>
> The Glb1 promoter comes from corn and therefore has been consumed safely by humans for thousands of years. No safety concerns have been identified with the consumption of DNA from GM plants. A typical diet contains DNA from many sources – bacteria, plants, and animals. It is highly unlikely the Glb1 promoter from corn poses any greater risk than any other piece of DNA in the human diet. As only one copy of this promoter has been inserted into LY038, this will not appreciably increase the amount of this element ingested as it is estimated that the entire novel DNA insert in LY038 represents only 0.0002% of the corn genome."

INBI: We are satisfied with this response so long as the Authority can verify that the "Glb1 promoter" is unaltered at the DNA sequence level.

INBI recommendation:

R.1 The Authority should report the DNA sequence of the Glb1 promoter in event LY038. Since the Applicant claims that it is the endogenous corn promoter, the actual sequence should not be a commercial secret.

FSANZ response to recommendation 7: Compositional analyses, NMR

> "The major nutrients and anti-nutrients present in corn have been identified by the OECD (OECD, 2002). These components were analysed in LY038 corn and non-transgenic control corn. In addition

to those components identified by the OECD, specific lysine related metabolites were analysed and compared between conventional and LY038 corn grain.

In addition to targeted compositional analyses mentioned above, profiling methods (such as metabolomic analysis) may be able to provide insight into metabolic pathways in the plant (ILSI, 2004). However, as has been observed in relation to compositional analyses, the large range of natural variation that occurs between plants can mean that a statistically significant difference between the test and control plants for any given nutrient may not necessarily be biologically significant. This is also a major challenge with the use of profiling techniques and therefore these techniques need to be validated and the range of natural variation clearly established before they can be used for the purpose of safety assessment (ILSI, 2004).

Currently the internationally accepted practise for evaluation of new GM plants relies on a variety of data and information, including compositional analyses, to identify any unexpected changes in the plant, which are then subject to further scrutiny to determine their biological relevance and potential impact on food safety. FSANZ considers these data and information, when considered in total, provide assurance that the food is unlikely to have an adverse effect on human health."

INBI: (1) Our recommendation resulted from a published demonstration of the effectiveness of this technique for profiling. The full ILSI paper referred to is not available to us. Our recommendation is repeated unless the ILSI specifically addressed, and criticized, *this* technique for profiling. (2) The Authority should elaborate on the variety of analyses used to identify unexpected changes, since in every other response to our submission it has attempted to justify the absence of methods for identifying unknown RNAs and proteins.

Please see Part Two, "Compositional analysis", for discussion and recommendations.

FSANZ response to recommendation 8: Allergenicity studies, pepsin resistance

"The Applicant conducted an in vitro digestibility study on the novel protein present in LY038 corn, cDHDPS, using a standardised protocol that has been shown to distinguish known allergens from proteins known not to be allergenic (Thomas et al., 2004). This protocol is not intended to be an exact replica of conditions in vivo, but rather is used to compare the test protein to known allergens under the same conditions.

The NZIGE object to the use of this protocol because the ratio of pepsin to protein is higher than would occur naturally in the human stomach and gastrointestinal tract. 10U of pepsin were used for every μg of test protein (2.64:1 ratio based on weight). Although in vivo protein levels will almost always exceed those of pepsin (Taylor, 2003), a standardized pepsin resistance assay is needed. For this reason the Applicant has used a protocol that has been shown to distinguish in vitro known allergens from non-allergens.

The recommendations of the WHO/FAO paper (2001) does not specify pepsin activity, but recommends an amount of pepsin based on weight. However, in reactions of this kind, enzyme activity is more relevant to the outcome than enzyme weight and for this reason, the protocol used by Thomas et al. (2004) is considered by FSANZ to be appropriate for assessing relative digestibility."

INBI: We are aware of the Thomas *et al.* paper and cited it in our original submission (7.1.10). This response does not address our concerns. 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). It also requires the use of standard control proteins which were absent in the experiments submitted by the Applicant (7.1.12). There is no scientific justification for the Applicant to pursue one test over the other, especially considering the FAO/WHO standard is the international consensus and the Thomas *et al.* standard is only the Industry preference. Therefore, the analysis should be done to both standards.

Please see Part Two, "Toxicity and allergenicity, pepsin resistance" for discussion and recommendations.

FSANZ response to recommendation 9: Amino acid levels

> "The reason the NZIGE has made this recommendation is elaborated on page 42 of the NZIGE submission. They are concerned by some apparent compositional differences (in amino acid levels) between the conventional corn lines used as controls in this study and the 99% tolerance interval from the conventional corn lines used as controls in study MSL-19172 (compositional analysis). Table 1 on page 42 of the NZIGE submission compares these values, however the data in the studies (MSL-18883 and MSL-19172) uses different units and therefore cannot be directly compared in this way.
>
> Once the amino acid values in question are expressed using the same units (% total amino acids) they fall within the 99% tolerance interval as shown in the table below" [not reproduced here].

INBI: We are satisfied with this response addressing confusion of the units in Table 1 of our submission. However, it is still unclear why the commercial corn lines used in MSL-18883 were not used in MSL-19172 (Compositional analysis). Surely the most complete analysis would have used varieties common to both studies. In MSL-18883, LY038 had higher levels in all of the 18 measured amino acids among the four commercial varieties used as references. In fact, the total amount of amino acids for LY038 was 115.17 mg/g compared to an average of 77.8 mg/g for the four commercial varieties.

We will elaborate on other concerns in Part Two, "Compositional analysis".

FSANZ response to recommendation 10: Nutritional impact, feeding studies

> "The study referred to in this recommendation is the broiler chicken feeding study. The NZIGE recommends dismissing this study due to confusion over the levels of amino acid in the control lines compared to those used in the compositional analyses. As discussed in Recommendation 9, these apparent compositional differences were due to differences in the units used.
>
> Study MSL-18883 is a comparison of different corn diets, including LY038 corn and conventional corn (either supplemented with lysine or not), in supporting the growth and performance of broiler chickens. This study was submitted by the Applicant to demonstrate the wholesomeness of LY038 corn. The rapidly growing broiler is considered to be sensitive to changes in nutrient quality in diets, and therefore is often used as a model to assess normal growth and well-being and the wholesomeness of corn.

> Normally, FSANZ does not require animal feeding studies to be submitted because when GM varieties have been shown to be compositionally equivalent to conventional varieties, feeding studies using target livestock species will add little to a safety assessment. In these circumstances the extent of the compositional data, molecular characterisation and toxicity / allergenicity data is considered sufficient to establish the nutritional adequacy and safety of the food. **In cases where the composition of food has been significantly changed, as is the case with high-lysine corn, feeding studies with suitable livestock species may be useful to confirm the wholesomeness of the food** [emphasis ours]**.**
>
> It is important to note that comparative feedings studies, like the one submitted for high-lysine corn, are not safety or toxicity studies and are only conducted with the purpose of demonstrating nutritional adequacy. Nevertheless, providing the study has been well conducted, the absence of adverse effects can provide additional assurances of safety. FSANZ has no valid reason to dismiss this particular study."

INBI: (1) The Authority is mistaken in its understanding of our reasons for concluding that the broiler study was too flawed to be considered valid for assessment of human food safety. (2) New Zealand and Australia are adherents of case-by-case assessment. Thus, generalities about past studies using different material do not substitute for properly conducted studies using whole food derived from LY038. (3) Given that high-lysine corn is a case of substantial nutritional change, and since FSANZ have chosen to defend the broiler study, we repeat our recommendations.

We were in part concerned about the amino acid levels. However, this was not the only reason we found the broiler study less than satisfactory (7.2.4-7.2.7). Broilers fed LY038 and derivative corn had significantly lower adjusted weight gain in the first 21 days 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. The Authority has not addressed this observation.

We also were troubled by the apparent contamination of LY038 seed stock by MON810, a different transgenic line. 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. 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. The Authority has not addressed this observation.

Please see Part Two "Nutritional impact, broiler studies" for recommendations.

FSANZ response to recommendation 11: Toxicity studies, animal models

> "As mentioned in the response to recommendation 10, FSANZ does not routinely require animal feeding studies to support the safety of a GM food. There are limitations in the extent to which such

comparative feeding studies can be used to detect adverse effects because there are constraints on the amount of test material (in this case, high-lysine corn) that can be incorporated into an animal's diet without creating nutritional imbalances. These studies are really only designed to demonstrate that the new GM food supports typical growth and wellbeing in the test animal. As a consequence, detailed blood analyses are rarely done.

These analyses have been requested by the NZIGE on the basis of a study in rabbits fed diets supplemented with various amino acids (Giroux et al., 1999). This study showed that diets high in lysine in combination with high levels of other amino acids led to increased serum cholesterol and phospholipids in the liver. Rabbits were fed diets containing 32.9 g lysine/kg diet, a very large amount of lysine compared to the levels found in LY038 corn (0.48% on a dry weight basis, or 4.8 g/kg dry weight). As the level of lysine in the supplemented diets given to the rabbits was very much higher than the levels found in high-lysine corn, this study is not considered relevant to the safety of high-lysine corn. In addition to this, corn is not a significant source of lysine in the human diet and even LY038 corn would not contribute significantly to lysine in the diet compared to other sources of dietary lysine.

There is no basis for the rationale that consumption of lysine from LY038 corn may have an effect on serum cholesterol."

INBI: (1) The Authority also said in their response to recommendation 10 that a valid feeding study was warranted in the case of high-lysine corn because it was substantially nutritionally different from conventional corn. (2) The Giroux *et al.* study was provided to the Authority to assist them in identifying a protocol for testing adverse affects. The Giroux *et al.* study did not preclude effects at lower concentrations of lysine than used, so this in itself is no reason to not do a comparable study with high-lysine corn. (3) The Authority has not addressed why they accept a level of reporting below that recommended by Renwick (Renwick, 2004). (4) The Authority has not addressed why they do not require a pig study of the type we recommend, especially considering that this would be far more suitable as model for humans than are chickens.

FSANZ response to recommendation 12: Toxicity studies, whole food feeding studies

"Long-term animal toxicity studies are not generally appropriate for the testing of whole foods. Such studies are commonly used in the safety assessment of discrete chemical compounds including pesticides, pharmaceuticals, industrial chemicals and food additives. In these cases, the test substance is well characterised, of known purity, of no nutritional value, and human exposure is generally low. It is therefore possible to feed such compounds to laboratory animals at a range of doses (using amounts greatly above expected human exposure levels) in order to identify any potential adverse effects. Establishing a dose response relationship is a pivotal step in toxicological testing. By determining the level of exposure at which no adverse effects occur, a safe level of exposure for humans can be established which includes appropriate safety factors.

In contrast, traditional toxicological testing is not intended to be applied to the assessment of whole foods. Foods are complex mixtures of constituents and have wide variations in composition and nutritional value. Due to its bulk, a food can only be fed to laboratory animals at low multiples of the amounts that might be present in the human diet and it is therefore not possible to conduct normal dose-response experiments in the same way that these experiments are conducted for medicines and chemicals. In addition, a key factor in these experiments is the need to maintain the nutritional value

and balance of the diet. A diet that consists entirely of a single food can cause adverse effects on nutritional status, potentially masking any other smaller effect of a component or components of the food being tested in the animals.

The observations from single food studies can therefore be confounded by a range of adverse effects not directly related to the food being tested.

Thus, a more focussed approached is required when the safety of a whole food is being considered. This approach is based on the principal that the safety of GM foods can be assessed by comparison to a conventional counterpart having a history of safe use, taking into account both intended and unintended effects. Rather than trying to identify every hazard associated with a particular food, the intention is to identify new or altered hazards relative to the conventional counterpart.

A component of this approach is to focus on the potential toxicity of any new proteins that have been introduced into the food through genetic modification. Because proteins are discrete chemical entities they can be fed to animals in large amounts, therefore it is possible to conduct animal toxicity studies to determine their safety. Acute toxicity testing has shown that the novel protein in LY038 corn, cDHDPS, is not toxic at high doses in rats.

However, a 3-month rat feeding study using LY038 corn was conducted by the Applicant and supplied to FSANZ. This study demonstrated no test substance adverse effects in rats fed up to 33% LY038 corn in their diets."

INBI: (1) Tests of the lengths that we recommend have been recommended by others in the peer-reviewed literature. (2) Animal feeding studies should not be dismissed because of the possibility that they could yield false positive results, especially considering that the alternative is to institutionalize potentially false negative indications of safety. (3) Our concerns are not just toxicological. (4) However, confirmation of the 3-month rat feeding study referred to above is welcome.

Please see Part Two, "Toxicity and allergenicity" for discussion and recommendations.

FSANZ response to recommendation 13: human tests

"The safety assessment for a GM commodity compares the molecular, toxicological and nutritional and compositional properties of the food to the non-GM form. The assessment focuses on the new gene product(s), including the intentional and unintentional effects of the genetic modification, and examines any compositional changes, including whether the genetic modification has altered the potential allergenicity and toxicity of the food. The assessment is therefore a comparative analysis using the commonly consumed conventional food as a benchmark for safety.

This comparative analysis is regarded by organisations such as the World Health Organisation (WHO)/Food and Agriculture Organisation (FAO), the Organisation for Economic Cooperation and Development (OECD) and the Codex Alimentarius Commission as the most practical approach for assessing the safety of a GM food. FSANZ regularly reviews procedures for assessment to ensure that recent scientific and regulatory developments are reflected in the process. At the international level, FSANZ is actively involved in the development of a framework for the assessment of GM foods within the Codex Alimentarius Commission.

> Although the NZIGE does not specify the human tests that they would accept, human studies are not considered an appropriate or necessary part of the safety assessment process for GM foods."

INBI: (1) We agree that FSANZ should benchmark with international food safety recommendations, but notes that FSANZ has accepted lower standards from submitted studies than recommended by these same bodies at several stages in assessing the safety of A549. (2) While we accept that FSANZ may choose not to require human testing, Codex Alimentarius does allow for more intensive pre-market testing for possible human-specific hazard identification beyond that provided by the Applicant.

(1) For example, FSANZ has accepted a study on cDHDPS digestion that is outside of UN FAO/WTO protocols. It has ignored Codex Alimentarius recommendations for testing using cooked and processed LY038 corn, and their recommendations that all novel proteins be isolated (refer to Part Two, "Characterisation of the genetic modification" and the 12 new open reading frames around the insert). In each case, the Authority has, in our view, relaxed adherence to international standards for safety testing when that better suited the Applicant's submitted work, and imposed international standards whenever that was a lower standard than we recommended.

(2) According to CAC/GL 44-2003 (p. 19 paragraph 48) "[F]oods derived from recombinant-DNA plants that have undergone modification *to intentionally alter nutritional quality or functionality* [emphasis ours] should be subjected to additional nutritional assessment to assess the consequences of the changes and whether the nutrient intakes are likely to be altered by the introduction of such foods into the food supply." Since LY038 is this type of modification, higher standards of review are justified.

FSANZ response to recommendation 14: Post market monitoring

> "GM food products are not permitted on the market if any question associated with negative health effects is left unanswered during the pre-market safety assessment. For this reason post-market monitoring is not considered necessary or useful as there is no potential adverse health outcome to monitor.
>
> Further, in Australia and New Zealand, as in most other countries, the responsibility for postmarket surveillance is covered by an ongoing duty of care on the part of the developer. The developer is expected to monitor for existing and emerging risks that may be associated with its product and notify regulatory authorities whenever new information is uncovered."

INBI: (1) The post-market monitoring of GM nutritionally enhanced foods is currently a subject of serious discussion in international food regulation, and FSANZ's regulation of LY038 should reflect this. (2) The Applicant's post-market monitoring plan has not been released for public comment.

(1) Codex Alimentarius states in CAC/GL 44-2003 that post-market monitoring may be appropriate "in specific circumstances". However, the necessity of post-market monitoring is not clear-cut internationally, especially for cases of nutritionally enhanced plants, such as LY038.

We highlight paragraph 31 of the Report of the Fifth Session of the Codex *Ad Hoc* Intergovernmental Task Force on Foods Derived from Biotechnology (ALINORM 06/29/34), which shows that post-market monitoring is a key consideration, particularly for nutritionally enhanced plants such as LY038:

> "The Delegation of the European Community, supported by some other delegations and observers, stated that considerations on post marketing monitoring systems should be an essential element of the work on this item because consumptions of nutritionally enhanced plants may cause significant changes in dietary intake patterns, in accordance with paragraph 20 of the Principle for the Risk Analysis of Foods Derived from Modern Biotechnology (CAC/GL 44-2003)."

With regard to continuing discussion at the international level, we support more stringent oversight of post-market monitoring as the direction of Codex Alimentarius becomes clear.

(2) Bearing in mind the Applicant's responsibility to undertake post-market surveillance, we assume that FSANZ has requested details from the Applicant on its post-market surveillance plans, to ensure that they exist and are of a suitable standard. We ask that this information be released. If these details have not yet been submitted to FSANZ, we recommend that they are requested now and are evaluated before a recommendation is made to the Ministerial Council.

Please see Part Two, "Reasons for post-market monitoring" for recommendations.

FSANZ response to recommendation 15: Food/feed

> "FSANZ is evaluating the safety for human consumption of food from LY038 corn. FSANZ does not have jurisdiction in relation to animal feed."

INBI: Recommendation 15 stated that FSANZ "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."

The answer did not properly address the issue we raised. FSANZ is indeed only mandated to assess food safety, and is funded and resourced with this in mind. However, in its Impact Analysis it included a statement related to the availability of LY038 animal feed for industry. This statement does not concern an impact of LY038 as a food ingredient. It refers to the import of LY038 as a separate product, and for a separate purpose, than the one being evaluated by FSANZ.

FSANZ's objectives and guidelines require it to take into account "the promotion of fair trading in *food*" and "the desirability of an efficient and internationally competitive *food* industry" [emphases added]. Nothing in FSANZ's objectives or guidelines asks it to consider implications for the animal feed industry.

For this reason, we are concerned that FSANZ does not have the ability, as a food safety body, to investigate the impact of animal feed availability or non-availability. As animal feed is outside FSANZ's mandate, we do not see how it can measure this impact. If the impact cannot

be measured, it cannot be considered to support either Option 1 or Option 2.

FSANZ response to recommendation 16: Impact analysis

> "FSANZ has conducted a comprehensive safety assessment on LY038 corn (Attachment 2 to the Draft Assessment Report), as it does on every new GM food and has not allowed the intention of the Applicant to segregate LY038 from the food supply to influence the rigour of the assessment."

INBI: FSANZ also stated in the DAR that it "is assessing LY038 corn as if it were intended to be consumed by humans. The safety assessment conducted on LY038 is as rigorous and thorough as for any GM food product, and assumes that if approved, corn from line LY038 could be routinely entering the food supply and not present just as an occasional inadvertent ingredient" (p. 13).

If the intention to segregate were disregarded, why were there references made to inadvertent contamination, or the likelihood of only small amounts of LY038 entering the human food supply, in the Safety Assessment or the Impact Analysis?

It is not clear why, if the intention to segregate were disregarded, FSANZ has continued to make reference to inadvertent contamination, or the likelihood of only small amounts of LY038 entering the human food supply, in the DAR. For example: "it is possible that a small percentage of LY038 grain will inadvertently be co-mingled with conventional corn and enter the human food supply" (p.8); "little LY038 will be entering the food supply" (p.11); "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 of products, or increased prices for non-GM food is likely to be low" (p.16); "because LY038 corn is not intended for food, human consumption is expected to be extremely low" (p. 31).

These references suggest that FSANZ has continued to evaluate LY038 in relation to the Applicant's current, declared intention rather than in relation to the potential impact of the approval. The statement that LY038 is being assessed "as if it were intended to be consumed by humans" is not substantiated by the nature of the investigations FSANZ has undertaken. We repeat: an approval will allow the legal import of LY038 as food, in whatever form or quantity the Applicant or Industry decide is appropriate in the future.

Please see Part Three, "Impact analysis" for discussion and recommendations.

FSANZ response to recommendation 17: Impact analysis

> "A 'split approval' process with different standards for products destined for animal feed to those destined for the human food chain has led to problems internationally when products unapproved for human consumption inadvertently entered the food supply. To prevent this occurring, FSANZ and the Office of the Gene Technology Regulator (OGTR) have a Memorandum of Understanding (MoU) that no 'split approvals' will be made. As the OGTR is responsible for the assessment and regulation of GM feeds, the MoU sets out an agreement that where a GM food product such as corn has not undergone safety assessment by FSANZ, the OGTR would not approve its use as animal feed until such time that it is shown to be safe for human consumption, through assessment by FSANZ.

> For this reason, FSANZ has agreed to assess the safety of this product for human consumption prior to it being used in animal feed. FSANZ has written the Regulatory Impact Statement to reflect this."

INBI: While we understand this regulatory arrangement, we seek further clarification on this issue because FSANZ has only mentioned the Australian regulatory system. FSANZ is a transnational regulatory body. The OGTR is an Australian body only, and nothing in this description pertains to the New Zealand regulatory system.

FSANZ response to recommendation 18: Impact analysis

> "FSANZ has an obligation to assess this GM food application and determine whether it would be appropriate to amend the Code to approve the use of food derived from corn line LY038 under Standard 1.5.2. In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives, which are set out in section 10 of the FSANZ Act. These objectives are also set out in Section 3 of the Draft Assessment Report on Application A549. Neither these three objectives nor the additional five points to which FSANZ must have regard specify that cost to government is a valid reason to reject an application. Furthermore, if FSANZ did not take this opportunity to assess high-lysine corn for its safety for human consumption and it were later detected in the food supply, the cost to government may be significant.
>
> FSANZ believes that the cost to consumers who wish to avoid GM products will not be significant even if this application were approved. Producers of high-lysine corn will aim to sell their product at a premium price as animal feed due to the high levels of lysine. It is less likely that food industry would pay premium price for high-lysine corn and therefore likely that the levels of high-lysine corn entering the food supply would be small. Further, food containing high-lysine corn is likely to be required to be labelled as GM and nutritionally different to conventional corn, allowing consumers to avoid this if they so chose."

INBI: (1) We question the Authority's reasoning in stating that it need not consider cost to government in its assessment. (2) In determining consumer impacts, FSANZ makes assumptions about the marketing of LY038 that overlook some significant possibilities, both deliberate and inadvertent, for its entry into the food supply.

(1) The Authority's argument that nothing in its objectives or guidelines "specify that cost to government is a valid reason to reject an application" implies that it does not need to investigate this issue.

However, this appears to be contradicted in the Authority's own summary of its own regulatory impact assessment: "Following a cost and benefit analysis of the potential impact of each of the options on the affected parties (consumers, the food industry and *government* [emphasis ours]), Option 2 is the preferred option as it potentially offers benefits to all sectors with little associated cost" (p.7). It is also contradicted within the FSANZ response to recommendation 18 itself, when it states: "if FSANZ did not take this opportunity to assess high-lysine corn for its safety for human consumption and it were later detected in the food supply, *the cost to government may be significant*" [emphasis ours]. FSANZ has a statutory requirement to consider the cost to government in its decision-making (FSANZ Act 13.2(c)), an impression strengthened by the fact that its Impact Analysis continues to refer to Government as an

Affected Party, and to government revenue and resource impacts as Impacts. Finally, it is not consistent with the Authority's approach to other proposed changes to the food code; for example, in the DAR for P293 it explicitly notes that "impact on enforcement costs for government" was one of the factors considered when determining its preferred regulatory option.

This Impact Statement referred to an impact on government monitoring resources for the implementation of labelling requirements. A quality regime of food safety and labelling relies on government funding and oversight. Without an assessment of the cost to government of monitoring and labelling, the oversight required to ensure "the provision of adequate information relating to food to enable consumers to make informed choices" (objective two), "the desirability of an efficient and internationally competitive food industry" (guideline three) and "the promotion of fair trading in food" (guideline four) cannot be guaranteed.

As previously stated, the need for a careful appraisal of monitoring and labelling costs comes from the fact that they are immediate and certain, rather than speculative, impacts of the proposed amendment to the food code. The implications should therefore be 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 that may be affected. 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.

Monitoring costs, both to industry and to government, are more than just possibilities. In order for this issue to be carefully considered, it is reasonable to ask for supporting information from FSANZ. Without such information, it is difficult to see how the Impact Analysis can be properly assessed.

(2) 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 reaffirm our position 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.

In Part Three, "Evaluation of the Impact Analysis, DAR, Option 2" we note several pathways through which it is reasonable to expect that LY038, if approved, will enter the food supply and, in doing so, will impose significant costs on consumers and food producers wishing to avoid LY038.

FSANZ response to recommendation 19: Special restriction on LY038 hybrids

> "Food from a hybrid plant line does not warrant a separate pre-market safety assessment if food from the parental GM plant lines have already been approved. FSANZ considers the food safety risks posed by the conventional breeding of GM plants are no different from those arising from the conventional breeding of non-GM plants. It is widely recognised that unintended changes may occur during conventional breeding, however the products of conventional breeding have a long history of safe use and are not regulated by FSANZ."

INBI: We have provided ample evidence in the case of LY038 to justify a restriction in Column 2 of the Table to limit this approval. However, if the Authority prefers, it could consider imposing a restriction with an expiry clause with conditions of review.

We will return to this topic in Part Two, "Reasons for special restrictions on any approval of LY038 in human food".

FSANZ response to recommendation 20: Cartagena Protocol

> "New Zealand has ratified the Cartagena Protocol on Biosafety, a multinational agreement to regulate the international trade of living modified organisms. The protocol is intended to help ensure that countries are themselves able to make decisions on import of living modified organisms to ensure their biodiversity is protected. Its main provisions relate to living modified organisms for intentional introduction into the environment (i.e. seeds for planting crops), although it has less extensive provisions relating to live modified organisms for food, feed and processing. If food derived from LY038 corn were to be approved by FSANZ, permission by other agencies in New Zealand (the Environmental Risk Management Authority) and Australia (the Office of the Gene Technology Regulator) would still be required before viable corn grain could be imported into either country.
>
> It is anticipated that if LY038 grain enters the food supply in Australia and New Zealand, it will be via processed imported food products."

INBI: We are satisfied with this response.

Part Two: Evaluation of the DAR

This section is a direct evaluation of the scientific documents submitted by the Applicant in support of A549, and FSANZ interpretations of the data. Additional detail may be found in the February 2005 NZIGE submission.

Characterisation of the genetic modification

Our recommendations are based on the evaluation of the study MSL-19109: "Amended Report for MSL-17770 "Molecular Analyses of Lysine Maize LY038" written by Mittanck, D.W., Rice, J.F., Palmer, G.M. and Reiser, S.E. (Monsanto Company 2004); and MSL-19871 "Molecular analysis of the LY038, LY038(-) Control and Inbred Maize Lines Contributing to the Genetic Background of LY038 and LY038(-)" written by Groat, JR, Wolff, BJ, Scanlon, NK and Masucci, JD (Monsanto Company 2005).

In the studies examined for the IAR, we raised the issue that the Southern blots used to conclude that

> "These results demonstrated that Lysine maize LY038 does not contain the cre cassette nor any associated partial or intact genetic elements" (p. 20) and "Results from these Southern blot analyses described below support the conclusion that the *nptII* cassette and any associated partial or intact genetic elements are absent in Lysine maize LY038 as no hybridisation signals were observed" (p. 19 Application, 24 October 2004)

were flawed because the comparisons were to another GM plant, thus what the Applicant referred to as nonspecific hybridization could not be distinguished from weak binding between probes and target sequences that were partial or re-arranged in both corn lines. The Authority has asked the Applicant to justify why it did not use a non-GMO control, preferably a parental line, in its molecular analyses[10]. The Authority rightly required the Applicant to address this issue. The Applicant replied with study MSL-19871 (dated 14 October 2005). Here we review that study.

Discrepancy in breeding histories

The origin of LY038(-) in the breeding schemes provided by the Applicant (e.g. figure 4 of MSL-19871 and figure 23 of the original application) remains confusing. In response to USDA questioning, the Applicant said that "Seed from a single ear on a single F1 plant were used to plant the F2 generation. The seeds on the F3 ears harvested from the F2 generation plants were planted in a separate row for each of the F3 ears. In the resulting F3 generation of plants, LY038 was selected from one of the aforementioned advanced ears, and the near isogenic control, LY038(-), was selected from another of the aforementioned advanced ears, based on its phenotypic similarity to LY038, and the absence of the LY038 trait based on event-specific PCR analysis" (p. 124)[11]. According to this, LY038 and LY038(-) were segregated at the F3

[10] Letter to Monsanto from Dr. Peter Abbott, FSANZ; dated 17 March 2005.

[11] USDA/APHIS Environmental Assessment in response to Monsanto Petition 04-229-01P Seeking a Determination of Nonregulated Status for Lysine Maize line LY038. http://www.aphis.usda.gov/brs/aphisdocs2/04_22901p_com.pdf. Access date 30 May 2006.

generation shown in figure 4 of MSL-19871), 2 crosses after the Cre-recombinase line was crossed to the progenitor line.

The Applicant replied to a similar question from the Authority[12] with the text they claimed they had also provided to the US FDA, but which is significantly different from what they told the USDA. "LY038 and LY038(-) progenitor plants were identified by selecting plants positive and negative for the linked *cordapA* and *nptII* genes based on *nptII* assay of intact plants before crossing with Cre recombinase-expressing plants in the F1' generation"[13]. The Authority should note that in this version of the breeding history, progenitors of LY038 and LY038(-) were already segregated before crossing to the Cre-recombinase line, about 3 generations earlier than reported to USDA (Figure 1).

We do not see how both of these histories can be correct. The significance of this observation is that the two sibling lines (LY038 and the negative segregant) would have bred separately for more generations in the FSANZ rendition than in the USDA rendition of the history, further reducing the already highly unlikely probability that LY038 and LY038(-) are more closely related to one another than either is to H99. (See also section "Compositional analysis", below, for additional discussion on this point.)

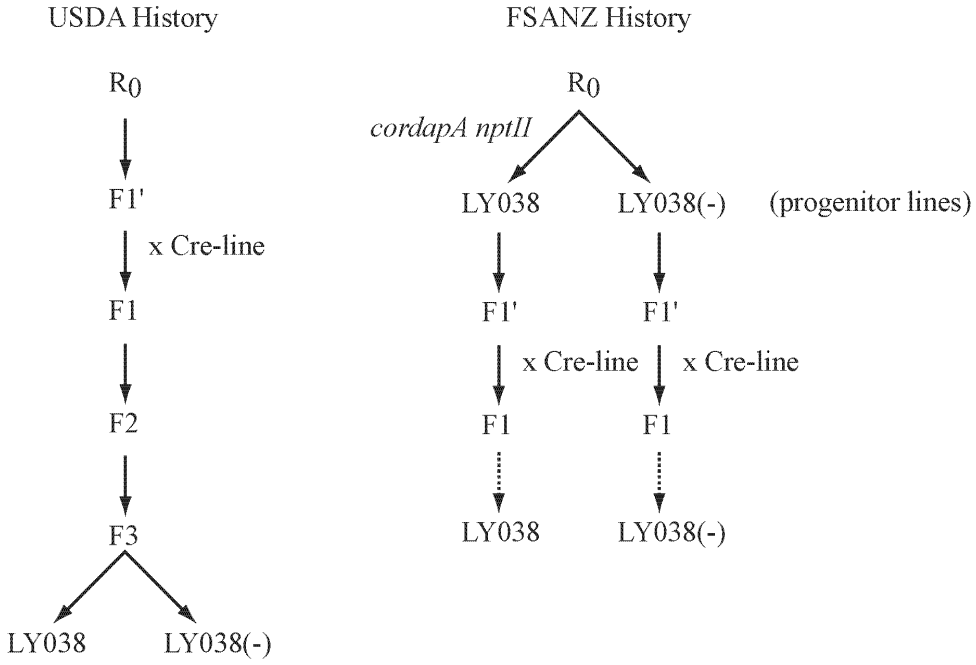


Figure 1: Breeding histories of LY038 and LY038(-).

We could find no explanation of the Applicant's response to FSANZ about the choice of controls. We can only assume that the Authority has chosen to exercise a standard that is lower than it could under CAC. The uncertainty that arises from the breeding histories undermines the strength of the conclusions derived in the molecular and the compositional studies, because they

[12] Email from Bronwyn Dixon (FSANZ) to Beth Bertuch (Monsanto), 11 March 2005.
[13] Email from Beth Bertuch (Monsanto) to Bronwyn Dixon (FSANZ), 11 March 2005.

rely on LY038(-) being the most closely related line.

R.2 The Authority should report the true breeding history for both LY038 and LY038(-) that includes the precise point at which the two lines segregate. From this history, the Authority should evaluate whether there is certain evidence that LY038 is more closely related to LY038(-) than to H99.

Sensitivity of Methods and Controls

Both the molecular characterization data from the original application (NZIGE submission sections 4-4.3.10) and in the supplemental study MSL-19871 fail to provide a description of probe sensitivity and investigator-determined stringency of washes. Such information is essential for drawing conclusions about the presence of insertions that are not full length or of identical structure (with regard to the probe DNA sequence). Partial fragments of I-DNA or genomic DNA interspersed into I-DNA have been detected as fragments as small as 15 bp in the peer-reviewed international literature (Makarevitch et al., 2003, Svitashev et al., 2002) (NZIGE submission section 4.3.5-4.3.7). The Authority should note that the residual *loxP* sequence would be about 34 bp.

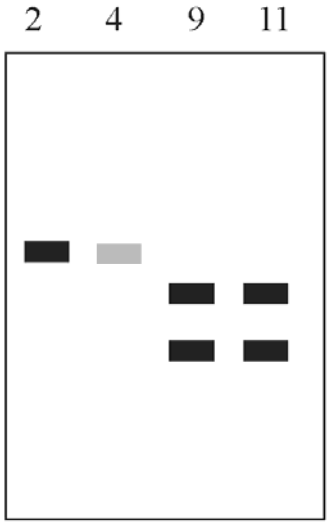
Consistent with CAC/GL 44-2003, "[t]he data and information, based on sound science, obtained using appropriate methods and analysed using appropriate statistical techniques, should be of a quality and, as appropriate, of quantity that would withstand scientific peer review." The Authority should hold the science of its assessment to this standard.

Study MSL-19871 updates the previous studies by including the parental stocks from which LY038 was derived. Consistent with the Applicant's claim, they can produce images of autoradiographs with identical patterns of "background" bands. There are two possible explanations for this. First, the background bands are actually limited to those composed of DNA sequences with fortuitous similarity to the probe (the Applicant's and the Authority's chosen explanation). Second, the Applicant has chosen a stringency of washes that removes probes bound weakly to small inserts composed of DNA corresponding to only part of the full length recombinant DNA used in transformation. This can be clarified by the Applicant revealing the sensitivity of their methods and positively identifying the sequence of all background bands detected at a sensitivity that would detect inserts significantly smaller than full length.

In figure 6 of MSL-19871, probe 2 was used to identify the *cordapA* gene inserted into LY038, verify that it was absent in LY038(-), and confirm that miscellaneous bands that hybridize in both LY038 and LY038(-) are explained by the genomic background of the two varieties. We have some concerns about this data. The inconsistencies are not easily explained by normal variation in the procedure. For example, we can see a weakly hybridizing band in lane 4 at about the 6.1kb mark (Figure 2). This lane has Inbred A genomic DNA. The hybridization signal is much less intense than in any other lane with a DNA fragment of the same size. This alone is not troubling, but, when coupled with the result in lane 11, it is. In lane 11, two bands are visible, each producing a signal that is far more intense than the single band in lane 4. How is this possible? How did the Applicant find more than twice the amount (and perhaps more

like 4 times the amount) of hybridizing DNA just by using different restriction enzymes?

Although not as clear cut, we have similar concerns about the pattern of hybridization intensities between lanes 8, with bands at about the 7.1kb and 0.7kb marks, and lane 15, with a band at about the 6kb mark. These lanes have Inbred B genomic DNA. The hybridization signal is much less intense than in any other lane, and the band is a different size than in the all other genomic samples. The hybridization signal of the apparent 6kb band in lane 15 is now as intense as any other band on the Southern. Moreover, it is a different size than in lane 8. All the other genomes produced two bands using this combination of enzymes, making Inbred B dissimilar in two ways from all the other genomes. The change in size may be expected since a combination of two different restriction enzymes was used, but the intensity should not change. Why did the Applicant not see equivalently intense hybridization in lane 8?



Lane numbers here correspond to lane numbers in MSL-19871. Lane 2: LY038 (-) (Spe I), Lane 4: Inbred A (Spe I), Lane 9: LY038 (-) (Xho I/Xba I), Lane 11: Inbred A (Xho I/Xba I).

Figure 2: Stylized cartoon of figure 6 from MSL-19871.

Too many easy errors in set up (e.g. loading the lanes with the wrong sample, sourcing DNA from mixed seeds) could have caused the results shown. If the results are not explained by simple errors in set up, then it suggests that they are dramatically affected by choice of probes and wash stringencies. Coupled with the extensive flaws in the other Southern blots submitted in MSL-19871 (see below), these unexplained anomalies leave us with little confidence in the data.

R.3 The Authority is requested to have the anomalous result in figure 6 of MSL-19871 explained, or have the analysis re-done, before accepting this as evidence of either a single insertion in LY038 or the absence of insertions in LY038(-).

In figures 5, 7 and 9 the Applicant claims that the observed diffuse hybridization near the top of the gel cannot be resolved as individual bands because of their high molecular weight. However, resolution of high molecular weight fragments can be achieved using pulse field electrophoresis, low concentrations of agarose, low running voltages and with the use of other restriction enzymes. Such techniques should be used to bring certainty to the interpretation of this Southern

because, as can be seen in, for example, figure 7, lane 3, at ~50kb there is evidence of another band that is unique to LY038.

The quality of figure 8 is of particular concern. In lanes 1-3 (LY038(-) and LY038) we observed faint, diffuse hybridization at ~35kb that is not seen for any of the conventional five inbred lines. In the previous molecular characterization study (MSL-19109), the blot was hybridized with probes that spanned the entire sequence of the PV-ZM003 plasmid (Figure 14, MSL-19109). No or only light background hybridization was observed. In this blot, a unique band was produced for LY038 DNA digested with *Spe I* (at ~3.8kb) and another unique band was produced with the digestion using *Xho I* and *Xba I* (at ~3.5kb). These results were explained as hybridization of the R-act1 promoter probe with the *cordapA* cassette containing the R-act1 intron. None of these bands were visible in Figure 8, creating concern in the reproducibility of the experiment, the suitability of the long probes and of the stringency of the washes used by the Applicant.

In both figures 8 and 9 the plasmid PV-ZM003 produced a single band that was larger than the expected plasmid fragment. This was explained by a possible difference in salt concentrations between the test substance DNA sample and the molecular weight marker. Although this explanation is plausible, it is challenged by the fact that none of these observations were made with plasmid PV-ZMPQ76 (Figure 5, 6 and 7) even though they were used with the same test substance, LY038(-).

Ambiguity about the amplification products in lane 3 of figure 22 of the original application remains. Lane 3 is DNA amplified using a primer specific to the I-DNA rAct1 intron. Up to 5 bands were generated from LY038(-). Under questioning from the USDA[14], the Applicant explained that the "Polymerase chain reaction with LY038(-) control DNA produced faint products; however, none of these products was the expected 4.1 kb in size." However, close inspection of the gel image reveals a band that does co-migrate with a band in the LY038 lane at approximately 4.1kb.

The Authority should be wary of the use of PCR to confirm the absence of other inserts. The anchor primer is to chromosomal DNA, and thus there is no guarantee from this analysis that inserts in other regions of the genome will be amplified efficiently if at all. Thus, there is no reason to dismiss the obvious implication of the amplification using LY038(-) DNA based on an expectation of the size of the amplified DNA. If the insert (partial or whole) were not precisely in the same place in both genomes, then the amplified products would not be the same size. This analysis only shows that an identified insert has an organization in the plant genome that is roughly similar to the organization it had in the original plasmid.

INBI recommendations:

R.4 Consistent with CAC/GL 45-2003, "the sensitivity of all analytical methods should be documented." Therefore, the Authority should report the minimum size of target DNA that all probes could detect at a minimum stringency of 0.5 copies per genome.

[14] USDA/APHIS Environmental Assessment in response to Monsanto Petition 04-229-01P Seeking a Determination of Nonregulated Status for Lysine Maize line LY038. http://www.aphis.usda.gov/brs/aphisdocs2/04_22901p_com.pdf. Access date 30 May 2006.

R.5 We recommend that the Authority require a range of analytical methods that includes a combination of FISH, fiber-FISH and Southern analysis.

R.6 The issue of background hybridization could be fully proved by sequencing the light bands visible in the Southern blots. The Authority should therefore base their final conclusion on the results of sequencing.

R.7 The Authority should clarify whether additional insertions are present in LY038 by requiring additional studies on the high molecular weight fragments in MSL-19871.

Antibiotic Resistance: Removal by Cre Lox recombinase

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) (NZIGE submission 4.3.8).

INBI recommendation:

R.8 The Authority should explain how it has confidence that the experimental procedures used by the Applicant would have detected an insert the size of the *loxP* site in an unknown location at 0.5 copies per genome.

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 or hybridization)]. In a study of tobacco plants, single *loxP* sites on different chromosomes mediated recombination between the chromosomes precisely at *loxP* in the presence of the cre recombinase (Qin et al., 1994). Thus, the Authority should be confident that all *loxP* sites in LY038 have been identified and eliminated.

INBI recommendation:

R.9 The Authority should verify that the residual *loxP* site in LY038 is not processed by the *cre* recombinase.

Untranslated RNA

See also "FSANZ response to recommendation 5", above.

The potential to inadvertently create novel RNA regulatory molecules, often 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., 2005) and (Hirotsune et al., 2003)) (1.3).

Since our last submission, the Applicant has now revealed that there are 12 new open reading frames around the insertion (DAR, p. 27). "The flanking corn genomic DNA was also sequenced. 1781 bp and 667 bp were sequenced at the 5' and 3' ends of the insert, respectively. Analysis of the sequence spanning the junction regions indicated that in the 6 reading frames at each junction…" As we previously argued, there is likely to be another open reading frame created by read-through of the nos terminator used by the Applicant (Rang et al., 2005), bringing the total to at least 13 known or likely new open reading frames.

New evidence indicates that dietary sources of dsRNA that have the potential to silence human genes can in certain circumstances be transmitted through food. dsRNA constructs produced in *E. coli* can effect gene silencing (RNAi) in the gut cells of mice (Xiang et al., in press). Mice were fed *E. coli* expressing dsRNA directed against genes in the intestinal cells. In this study, the bacteria were nonpathogenic but engineered to invade human cells. Although this demonstration was not the same as feeding naked dsRNA, the Authority should also be aware that the dsRNA in LY038 would be protected by the plant cell or cellular debris, or the surface of bacteria, and therefore could very likely survive the stomach.

According to CAC/GL 44-2003 (p. 14, paragraph 32), "Information should be provided on any expressed substances in the recombinant-DNA plant; this should include: the gene product(s) (e.g. a protein or an untranslated RNA); the gene product(s)' function…". Regardless of whether the variant RNAs arise from a cryptic splice site within nos, through other processing pathways, or from newly created open reading frames, all novel RNA species in LY038 must be reported and tested for a proper safety assessment.

INBI recommendations:

R.10 The Authority should provide evidence that all novel RNA species have been identified, characterized and tested for food safety.

R.11 We recommend that the Authority require a complete microarray description of the LY038 transcriptome, compared to the unmodified control, for proper hazard identification.

R.12 The Authority should require the Applicant to report on results of microarray analyses using the mouse genome and RNA extracts from the intestinal cells of mice fed LY038.

The Applicant goes on to say that "As mentioned in Section 3.3, only one novel open reading frame starting with a methionine codon and of significant size (>100 amino acids) was identified. However, bioinformatics analysis of this and the other 11 putative open reading frames was performed using the ALLPEPTIDES, TOXIN5 and AD4 (the allergen database)

databases. Analysis was also done on the putative polypeptides encoded by reading frames two to six of the cDHDPS protein coding sequence of the insert. No biologically relevant structural similarities to allergens, toxins or pharmacologically active proteins were observed for any of the putative polypeptides" (DAR, p. 37).

The Applicant has imposed criteria on its bioinformatic characterization that are not universal. For example, not all proteins begin with the AUG codon. Futhermore, FSANZ has the option, under Codex Alimentarius, to request biological data. According to CAC/GL 44-2003 (p. 14, paragraph 32),

> "Information should be provided on any expressed substances in the recombinant-DNA plant; this should include: the gene product(s)' function…" and (p. 22 Section 2) "As there is no single test that can predict the likely human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. *This will require the isolation of any newly expressed proteins from the recombinant-DNA plant*, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and biochemically equivalent to that produced in the recombinant-DNA plant. *Particular attention should be given to the choice of the expression host, since post-translational modifications allowed by different hosts (i.e.: eukaryotic vs. prokaryotic systems) may have an impact on the allergenic potential of the protein*" (emphasis ours).

The Applicant has produced no biological demonstration that the additional putative peptides are biologically irrelevant. This uncertainty could largely be addressed through the conduct of a proper animal feeding study using material from whole plants cooked and processed as in human food preparation. Moreover, there is now considerable evidence that small peptides (<100) can have many important biological roles but these proteins are systematically under-reported in the literature (Kastenmayer et al., 2006).

> "Knowledge of sORF (small open reading frame; <100 amino acids) function is limited compared to that of larger genes, although small proteins include members of important classes such as mating pheromones, proteins involved in energy metabolism, proteolipids, chaperonins, stress proteins, transporters, transcriptional regulators, nucleases, ribosomal proteins, thioredoxins, and metal ion chelators (for review, see Basrai et al. 1997). Computational discovery of sORFs is difficult because they are "buried" in an enormous pile of meaningless short ORFs that arise by chance. In addition, sORFs are not favorable targets for random mutagenesis. Similar challenges plague attempts to identify non-coding RNAs (ncRNAs), transcripts that function at the level of RNA rather than as templates for translation (for review, see Eddy 2001). Despite the challenges of sORF identification, reports since the publication of the *S. cerevisiae* genome indicate that sORFs are quite numerous in *S. cerevisiae* and many are evolutionarily conserved from distantly related fungi to humans" (Kastenmayer et al., 2006).

INBI recommendation:

R.13 While the Applicant continues to rely upon unvalidated methods (e.g. bioinformatics as described above) for hazard identification, the Authority should make the insertion and flanking sequences publicly available for evaluation by those who may then bring more relevant analyses to bear.

Food Processing

According to CAC (p. 18 paragraph 47) "[t]he potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food ingredient from the plant." These criteria apply to LY038 because it has an enhanced potential to create toxic, harmful and anti-nutrient products via the Maillard reaction. It would be valid in a case-by-case assessment to require food processing studies for LY038. (See also Part One, "FSANZ response to recommendation 3", above).

AGEs are linked to cancer, allergens and adverse health effects

Maillard reactions are non-enzymatic browning reactions which occur between amino acids and carbohydrates (particularly reducing sugars). Amadori products are created in the first stage of the reaction through condensation between a free amino group, usually of lysine, and a carbonyl group of a reducing sugar (Gerrard, 2006, Henle, 2005). "Depending on time and temperature during heating or storage, up to 70% of lysine initially present in proteins may react to the Amadori product" (Henle, 2005). These reactions are, in fact, a series of parallel reactions that are "influenced by each other as well as by milieu parameters" (Henle, 2005).

Amadori products may undergo several degradation reactions in foods leading to the formation of 1,2-dicarbonyls (Henle, 2005). These species are highly reactive with proteins and lead to formation of late Maillard products. Therefore, the compositional elements from corn relevant to the safety assessment of Maillard reaction products are proteins, free amino acids (particularly lysine) and carbohydrates (particularly reducing sugars).

In biological systems, "glycation" products including carbonyl derivatives and advanced glycoxidation endproducts (AGEs) are produced by corresponding Maillard reactions (Akagawa et al., 2005, Gerrard, 2006, Henle, 2005). Dietary AGEs are thought to contribute "to the pathologic sequelae seen in normal aging, diabetes, and kidney disease" (Goldberg et al., 2004), including wound healing retardation (Peppa et al., 2003a) in diabetics, and neurodegenerative diseases such as Alzheimer's (Elliott, in press). Recently, a link between lysine AGEs and cancer was made (Heijst et al., 2005). Higher levels of AGEs are detected in Creuzfeldt-Jacob Disease (CJD) patients, but it is unknown whether they are a symptom, side-effect or contributor to the disease (Freixes et al.). Glycation can alter the longevity of peptides in the intestine; this stabilization of proteins has implicated glycoxidation with diabetes related autoimmunity (Elliott, in press). There is also evidence that some AGEs may be beneficial (references in Henle, 2005).

Glycation products have a low resorption rate (Henle, 2005), thus glycation of lysine and protein reduces the nutritional value of the food while increasing the stability of the protein and, concomitantly, the potential for glycation products to become allergens. The very latest research indicates that some allergens are attenuated or removed by heat or during processing, but other allergens become more potent as a result of heating and in the presence of

carbohydrates (Gruber et al., 2005). These can only be identified using food prepared in a fashion representative of how people will consume it.

> "In contrast to these so-called pollen-related allergens, roasting has been reported to increase the allergenicity of raw peanuts (10). For example, protein extracts of thermally treated peanuts have been shown to bind IgE antibodies from patients' sera at up to 90-fold higher levels than extracts obtained from the corresponding nontreated peanuts (10). In addition, inhibitory ELISA experiments revealed a significant increase in the IgE binding activity of the purified major allergens Ara h 1 and Ara h 2 after thermal treatment in the presence of carbohydrates (Gruber et al., 2005)".

In this example, even the minor allergen Ara H 1/2 (peanut agglutinin) was converted into an IgE-binding product after incubation with sugar at elevated temperatures (Gruber et al., 2005). These results clearly indicate that the allergenicity of cDHDPS and other proteins in LY038 or its derivatives cannot be identified by past experience feeding animals uncooked and unprocessed sources of corn.

There is overwhelming scientific reason (presented in the following paragraphs) to believe that LY038 composition is likely to yield Maillard reaction products, including anti-nutrients and AGEs, and that it will produce these compounds in higher quantities than conventional corn and many other foods with comparable total lysine.

LY038 is substantially different in composition to conventional corn with respect to the potential to form food hazards.

For food, milieu parameters effecting Maillard reactions will include the availability and concentration of reactants (both free amino acids and those in proteins, and reducing sugars present in carbohydrates) and exposure to elevated temperatures. These are the milieu elements relevant to the product being considered—LY038 and its derivatives—that have been modified to produce high endogenous levels of lysine, because one reactant (lysine) is at unprecedented concentrations in the milieu of reducing sugars characteristic of the corn grain.

Our analysis of the Table provided on p. 65 of the DAR revealed four important points.

1. The "control corn grain" for which a lysine level is reported (320 mg/100g) is not a valid control. CAC/GL 44-2003 (p. 2 paragraph 4) does not allow another GM crop to serve as a control, because neither crop has a history of safe use.
2. LY038 corn has the potential to significantly alter AGE intake in the Australia/New Zealand diet. High lysine corn would provide amounts of free lysine comparable to animal sources of free lysine (in absolute amounts; Table 1 column 5). LY038 would thus eliminate corn as a source of calories and nutrients that would be expected to be low in AGE content and endanger recommendations to encourage consumers to eat more vegetables instead of meat.
3. Generally speaking, it is not informative to compare absolute lysine levels when reviewing the potential effects of AGEs because those conventional foods with high-lysine levels are extremely low in carbohydrates (Figure 3). Moreover, foods high in lysine content are usually extremely low in free lysine (Table 1).

4. The ratio of free lysine to total lysine is profoundly different between LY038 and all other foods we could find comparisons to (Table 1, column 4). The ratio is 31, 47, 19, 28 and 70 *times* larger in LY038 than in LY038(-), sweet corn, lentils and various species of fish, respectively.

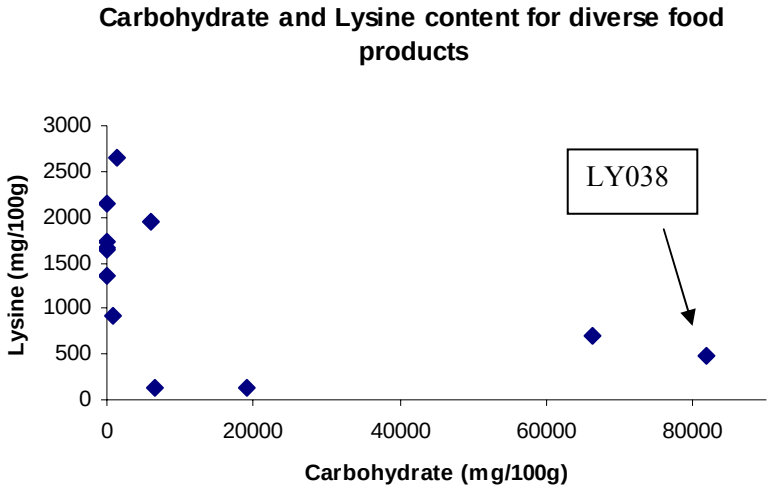


Figure 3: Lysine and carbohydrate relationships in common foods and LY038

Table 1: Comparisons of free lysine in common foods and LY038

Food	Free Lysine (mg/100g)	Total Lysine (mg/100g)	Free/Total Lysine (%)	Annual Free Lysine (g) Consumption[15] Australia/New Zealand	Free Lysine Reference
Corn, LY038	135	480	28	7.2/3.3	
LY038(-)	3	320	0.9	0.2/0.075	
Lentils (raw) cotyledon seedling	13* 30*	1957	0.6 1.5		(Rozan et al., 2000)
Fish[16] Mahi-mahi flounder bigeye tuna	53 17 8	N/A 1731 2147	N/D 1 0.4	11.7/14 3.8/4.5 1.8/2.1	(Antoine et al., 1999)

*fresh weight

LY038 cannot be compared to other varieties of corn or other foods because other varieties of corn and other foods have much lower levels of free lysine (Table 1). Vegetables normally are low in free amino acids, especially lysine (Mennella et al., 2006). There is approximately 52 times the amount of free lysine in LY038 in comparison to the Applicant's control[17]. The ratio of free lysine to total lysine is also significantly different in LY038 and the Applicant's control; 28% of lysine in LY038 is free lysine, vs. only 0.9% in LY038(-) (Table 1). Maillard reaction products and reaction rates can differ between free lysine and lysine in protein because the context of lysine in the peptide influences reactivity (Mennella et al., 2006).

LY038 cannot be compared to non-corn foods because non-corn foods with higher lysine levels have much lower levels of carbohydrates. Conventional corn will likely have different concentrations and types of carbohydrates available to react with lysine than do red meat, eggs, cheese or fish, or any of the products in the table[18] provided by FSANZ (Table 2[19]).

LY038 must be found to be as safe as conventional corn, not other conventional foods. The goal of the assessment is to determine whether people could source 100% of their normal intake of corn as LY038. The goal is not to compare the health trade-offs of eating LY038 corn and roast lamb. Because the Maillard reaction products we discuss are milieu-specific, the table

[15] Based on annual per capita corn consumption of 2.5kg in New Zealand and 5.3kg in Australia. Source FAOSTAT 2006.

[16] Based on annual per capita fish consumption of 26.3kg in New Zealand and 22.1kg in Australia. Source FAOSTAT 2006.

[17] Comparison from Table 14 of MSL 19172.

[18] DAR p. 65

[19] Unless specified otherwise, source is USDA Nutrient Data Laboratory http://www.ars.usda.gov/main/site_main.htm?modecode=12354500. Access date 29 April 2006

of lysine content for different kinds of foods is immaterial to this risk assessment.

Table 2: Carbohydrate and lysine content by food

Food[20]	Lysine content (mg/100g)	Carbohydrate content (mg/100g)
Corn, LY038	480[21]	81800[22]
Oats	701	66270
Corn, sweet	137	19020
Broccoli (raw)	135	6640
Lentils (raw)	1957	6080
Cheese (edam)	2660	1430
Egg (raw, fresh)	914	770
Chicken (Chicken, broilers or fryers, back, meat only, raw)	1661	0
Fish (Pacific cod, raw) Fish, flatfish (flounder and sole species), raw Fish, tuna, fresh, yellowfin, raw	1644 1731 2147	0 0 0
Red meet (Beef, chuck, blade roast, separable lean and fat, trimmed to 1/2" fat, prime, raw)	1359	0

LY038 hazards will be seen only after cooking.

It is justified on scientific grounds to expect the production of undesirable AGEs or anti-nutrients in LY038 above and beyond that found in conventional corn after heating, processing or cooking. AGE content in food increases with cooking time and temperature (Elliott, in press, Goldberg et al., 2004, Henle, 2005). "The amount of AGEs present in all food categories [including vegetables] was related to cooking temperature, length of cooking time, and presence of moisture" (Goldberg et al., 2004).

The use of infant formula has been associated with a rise in childhood autoimmune diseases. Infant formula (e.g. Enfamil) already contains corn (7.1.9) and corn-derived products and are already known to be 100-fold higher in AGE content than human or bovine milk (Goldberg et al., 2004). There can be no justification for increasing higher concentrations of glycation reactants in infant formula, as an amendment to the Food Code would allow.

In our previous submission, we highlighted the relationship between free asparagine, sugar and heat in producing acrylamide by the Maillard reaction in cooked food. This relationship is increasingly well documented and attracting significant concern.[23] In a seminal study of french fries made from 66 potato varieties that differed in sugar and free amino acid content, Becalski

[20] Unless specified otherwise, source is USDA Nutrient Data Laboratory http://www.ars.usda.gov/main/site_main.htm?modecode=12354500. Access date 29 April 2006

[21] Source Table 11 of Appendix IV Monsanto Application, October 2004

[22] Source Table 14 of MSL 19172

[23] http://www.sciencenews.org/articles/20021214/food.asp. Access date 3 May 2006.

et al. (2004) found (Figure 4, redrawn from original) that "the formation of acrylamide is favored by an excess of sugars" in potatoes (Becalski et al., 2004). They conclude for this Maillard reaction that "our study shows the importance of controlling the precursors of acrylamide" for controlling the accumulation of acrylamide in food. There is no reason at present to suspect that lysine and sugar reactants that produce other potentially harmful AGEs would differ from these findings with acrylamide.

According to Science magazine[23], "[N]one of these data even start to indicate whether the concentrations of acrylamide now present in the human diet pose a risk to health at the amounts currently being consumed. *Such an assessment will probably require rodent-feeding trials with a range of intakes representative of different population groups*" [emphasis ours]. Why is the Authority content with an application that does not attempt to measure well documented potential harms using an appropriate animal feeding study? Asking for such data would be consistent with international food safety standards as set out by the CAC.

Historical evidence of anti-nutrients

There is historical evidence that lysine formed anti-nutrients by the Maillard reaction in corn (Panigrahi et al., 1996), which means that lysine in corn cannot be generally regarded as safe (GRAS) in this particular case. Broiler chicks fed corn with varying levels of stackburn were found to receive up to 14% less nutritive value than chicks fed control corn. Stackburn is the result of Maillard reactions occurring during corn storage. It occurs at temperatures much milder than those that may be used during processing and cooking for human foods.

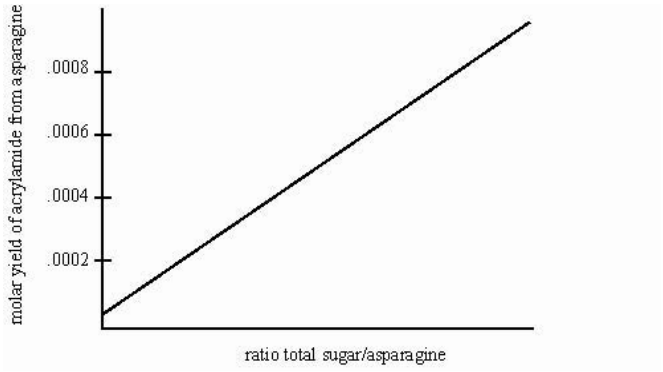


Figure 4: Acrylamide formation in cooked potatoes

Lysine is the major reactant in the formation of Amadori products in stackburn corn, with up to half of the lysine converted (Panigrahi et al., 1996). Under these mild heating conditions with conventional corn—of inherently low lysine content—there was no evidence of short term toxicity from the Maillard products. There was, however, demonstration of a significant anti-nutrition effect and a corresponding decline in metabolizable energy with increases in browning.

LYO38 has the potential to augment the AGE content of processed food and elevate the risk of AGE-related adverse effects

LY038 has elevated levels of pipecolic acid and total lysine, and unprecedented levels of free lysine, saccharopine and α-aminoadipic acid (Table 3). All of these compounds and cadaverine can react with reducing sugars to form Maillard reaction products (AGEs) (e.g. Akagawa et al., 2005). In addition, α-aminoadipic acid has a neurotoxic activity (Rozan et al., 2001), pipecolic acid may incite chronic hepatic encephalopathy, and cadaverine augments histamine toxicity. There is overwhelming reason to suspect that LY038 will produce an entirely unique spectrum of food hazards when cooked or processed. These hazards will be completely beyond what can be predicted from raw LY038 corn or raw or cooked conventional corn.

While vegetable sources probably contribute the least amount of AGE content in the diet, LY038 and its derivatives have the potential to boost exposure from all foods that have a corn component, including many processed foods which are heated to high temperatures. "Processing of some ready-to-eat cereals, which includes heating at temperatures over 230°C, may explain the high AGE content of these products. Also, many cereals and snack-type foods undergo an extrusion process under high pressure to produce pellets of various shapes and densities. This treatment causes major chemical changes, thermal degradation, dehydration, depolarization, and recombination of fragments all of which can promote glycoxidation" (Goldberg et al., 2004).

Table 3: Maillard reactants and factor increase in LY038

Reactant	LY038 (μg/g)	Control (μg/g)	Factor increase
α-aminoadipic acid	56.59	<5	≥10
saccharopine	650.29	5.88	110
free lysine	1351.13	25.99	52
cadaverine	<5	<5	unknown
L-pipecolic acid	28.72	14.96	1.9

We cannot see how it is in the interests of the people of New Zealand or Australia to augment the AGE content potential of processed foods or infant formula. There is no scientific justification for not testing the effects of this transgenic material under conditions that approximate the forms in which LY038 products will most often be consumed by people.

INBI recommendations:

R.14 The Authority should report not just total lysine content of foods, but free lysine content of foods and provide comparisons with conventional corn, especially H99. The Authority should also consider the ratio of carbohydrate to free lysine.

R.15 The Authority should provide the people of Australia and New Zealand with reliable data demonstrating that processing and cooking temperatures normal to products that could contain this corn are as safe as products derived from conventional corn, particularly the parental varieties of LY038.

R.16 The Authority should request an analysis of all novel AGE content or AGE concentrations, including Maillard reaction products and glycotoxins, that could arise from cooking, storage or processing of LY038 corn compared to parental varieties.

R.17 The Authority should justify its conclusion that lysine levels in a genetically modified variety of corn can be considered safe by comparison to lysine levels in unrelated food sources, such as red meat, chicken, eggs, cheese, broccoli, lentils and fish.

R.18 The Authority should require 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, and compared to the parental varieties.

R.19 The Applicant should conduct dietary AGE mouse feeding studies equivalent to those reported by Peppa *et al.* (Peppa et al., 2003b).

R.20 The Authority should justify its claim with reference to recommendations of international food safety agencies that for LY038, with its significantly different nutritional profile, additional feeding studies are not required.

Compositional Analysis/Comparative Analysis

Our recommendations are based on the evaluation of the study MSL-19172 : "Compositional Analyses of Forage and Grain collected from Lysine Maize LY038 grown in the US Field Trials in 2002" written by Reynolds, T.L., Nemeth, M.A., Fuhrman, J.D., Trujillo, W.A. and Sorbet, R . (Monsanto company 2004).

Choice of control maize and statistical relevance of results

The Applicant must make comparisons to proper controls, what CAC calls 'conventional counterparts'; "a related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food" (CAC/GL 45-2003, p. 1 paragraph 8). "It is recognized that for the foreseeable future, foods derived from modern biotechnology will not be used as conventional counterparts" (CAC/GL 45-2003 p. 1 footnote 5). Additionally, the OECD recommends that "measurement data from the new variety should ideally be compared to those obtained from the near isogenic non-GMO line grown under identical conditions" (ENV/JM/MONO(2002)25 p.18) [24]. In FSANZ's instructions to Applicants (Format[25]) (p. 6), the Authority advises that "normally, [the comparator] would be the near isogenic line or strain that was transformed to produce the GM line or strain (i.e the parental line). Where this is not possible, the comparator should be as close as possible to the GM line or strain."

In this case, the most suitable control was both the parent and "the near isogenic line or strain that was transformed to produce the GM line", H99, because it was not a product of gene technology and it is more closely related to LY038 than is LY038(-). LY038 shares more than

[24] When referring to ENV/JM/MONO(2002)25, we make specific reference to the Consensus Document on Compositional Considerations for New Varieties of Maize (*Zea Mays*): Key Food and Feed Nutrients, Anti-Nutrients and Secondary Plant Metabolites from OECD found at http://www.oecd.org/document/9/0,2340,en_2649_34385_1812041_1_1_1_1,00.html Access date 2 May 2006.

[25] http://www.foodstandards.gov.au/_srcfiles/Application%20Format%20-%20GM%20June%2005.doc. Access 29 May 2006.

65% of its genome with H99. LY038 and LY038(-) are siblings. Mendel's law of independent assortment means that LY038 and LY038(-) are as likely to be 100% related as they are to be 0% related. On average, siblings have 50% identity. What we can say with near certainty is that LY038(-) does not have at least one chromosome that LY038 has, the one with the *cordapA* insert. Depending on whether LY038 is homozygous or heterozygous, LY038(-) and LY038 must differ by at least ~10% or 5%, respectively.

There is nothing in the breeding histories to tell us that LY038 and LY038(-) were screened beyond the characterization that LY038(-) does not have the *cordapA* gene, so we cannot say how many chromosomes from H99, Inbred A, B, or C, that each sibling has from these parents they also hold in common. They may have more, depending on how many of the same chromosomes from the parentals each holds in common, and may in fact have *no* chromosomes in common. In the absence of data, the Authority can only assume that, on average, siblings will be 50% identical.

That makes H99 the closest related corn. Moreover, it is also a non-GM parental, making it without question the suitable comparator line for the molecular characterization and the compositional analysis. Inbred D, which is not in the parentage of LY038 (Figure 2), is the closest relative of LY038(-).

Instead, in the Authority's own words, "[t]he Applicant has provided information comparing LY038 corn to a closely related control corn crop, LY038(-), both grown in the same location" (DAR p. 11). Why has the Authority accepted LY038(-) as a comparator in the molecular and compositional studies? The Authority's recommendation appears to be based on data derived from a control strain that is outside Codex Alimentarius recommendations and FSANZ policy.

Both parentage and environment contribute to the variance in phenotypes measured for the compositional analyses (Reynolds et al., 2005). In the Applicant's words: "The large number of statistically significant differences in the levels of these analytes between seven commercial hybrids emphasizes the importance of genetic background and environment as determinants of the biochemical composition of maize grain" (Monsanto study published under Reynolds et al., 2005). Geneticists have been aware of the influence of genotype on phenotype for about a century (Heinemann and Roughan, 2000), and thus put great weight on using isogenic series of organisms when trying to determine the effects of single genes or mutations. The isogenic series would normally be composed of the parental genotype and the recombinant derivatives.

The Applicant has not used a non-GMO isogenic series to draw conclusions about the composition of LY038 and conventional corn. By not making measurements between the parental varieties of LY038 and LY038 in a series of five environments, the Applicant may inflate the variation and mask potential hazards. The Applicant has possession of the non-GMO parental lines (H99, Inbred A, B, C and possibly D). For these reasons, the Authority should expect the Applicant to provide the comparator data in the format specified by the Authority.

The Applicant has also made one-off measurements (with internal replicates) of four different non-GMO varieties per site and compared the range of those measurements to LY038. Variability among measurements of constituents in the non-GMO varieties is proportional to

the diversity of their genotypes within each test site. The only comparison thus provided to the Authority was between LY038 and LY038(-) at a site and the average and variance of 18 distantly related varieties grown at 5 different sites.

From the data provided, it would also appear that the compositional measurements at each site were made within a single year. This is also outside the standards set by FSANZ in their advice to Applicants. The Authority advises that "trials should be over a sufficient number of years to allow adequate exposure to conditions met in nature" (Format p. 6).

The Applicant changed both the environment and the composition of genotypes by planting four different non-GMO "reference" varieties at each of the different sites. The combined data then produces an overwhelming variance range because it is a composite of a few replications of each genotype, each genotype grown in only one environment, and five different environments. It is improbable, if not impossible, with this type of experimental design to recognize and eliminate outliers or produce useful baseline data for hazard identification.

This is also contrary to the format specified by the Authority (Format, p. 6) where it says that "[t]he pooling of data from different sites is acceptable provided data from the separate sites is also submitted and separately evaluated", which it was not in A549. In the statistical analysis, the range of observed values for the reference substances was always a combined value across all sites. There was no reference range reported by site.

Natural variability is not a baseline for analysis, despite what the Applicant repeatedly claims (e.g. Reynolds et al., 2005). Hazard identification is the baseline for analysis. Thus, minimizing non-specific variability should be the goal of a risk assessment. This is most reasonably done using the proper non-GMO parental lines as controls.

The Authority has asked the Applicant to justify why it did not use a non-GMO control in its compositional analyses, and why it did not use the parental varieties as controls in its compositional analyses[26]. We could find no explanation of the Applicant's response to FSANZ. We can only assume that the Authority has chosen to exercise a standard that is lower than it could under CAC. No cogent rationale has been offered to the people of New Zealand and Australia by the Applicant or the Authority as to why the Authority should accept a lower standard than it could under international guidelines.

INBI recommendations:

R.21 The Authority should explain why it has accepted comparisons between LY038 and another product of gene technology with no history of safe use, LY038(-), rather than the CAC recommended standard of a comparison to conventional parental varieties.

R.22 The Authority should explain why LY038(-) was used as a control instead of the more closely related conventional variety, and parent, H99.

R.23 If the Authority accepts LY038(-) as a control, then it should explain how it verified the absence of small inserts in LY038(-) with experiments that would detect the 34 bp *loxP* sequence at 0.5 copies per genome.

[26] Letter to Monsanto from Dr. Peter Abbott, FSANZ; dated 17 March 2005.

R.24 The Authority should provide a statistical analysis of the reference ranges per site.

R.25 The Authority should base its recommendation to amend the Food Code based on a proper comparison between LY038 and its parental varieties H99, Inbred A, B, and C grown under identical conditions in at least five test sites repeated in at least two growing seasons.

Choice of conventional counterparts and references

Since the Applicant has provided compositional studies with flawed controls, notably the improper substitution of a GM corn variety [LY038(-)] for a conventional counterpart, we recommend that the Authority consider substituting formal OECD historical ranges for proximates (ENV/JM/MONO(2002)25 p.20), reproduced in Table 4.

Our analysis of Table 4 revealed two important points.

1. The value of protein content, TDF, ADF and NDF for LY038 is outside the OECD range. "If the characteristic level for a specific component, which is altered in a specialty type of maize, is outside the general range of values found in scientific literature, the comparison with the parent line will be decisive" (ENV/JM/MONO(2002)25 p.19). Therefore the Authority must ask the Applicant to provide data using the parental lines H99, Inbred A, B, and C.
2. Some of the reference maize range values are significantly outside the OECD range. This observation again leads us to question how the Applicant came to choose specific lines for this study, and whether or not those reasons were consistent with a design to optimize the analysis of LY038 as a human food.

Table 4: OECD Proximate Analysis

	LY038 value	Range from OECD Table 2	Range of reference maize Table 5 from DAR
Ash	1.44 ±0.033	1.1-3.9	1.05-1.75
Carbohydrates	81.8 ±0.62	82.2-82.9	80.26-87.96
Moisture	8.91 ±0.40	7-23	7.68-11.1
Protein	**12.90 ±0.56**	6-12.7	7.61-14.69
Total fat	3.86 ±0.20	3.1-5.8	2.03-4.53
TDF	**20.77 ±2.48**	11.1	**12.58-35.31**
ADF	**6.57 ±0.42**	3.0-4.3	4.29-9.56
NDF	**12.56 ±1.08**	8.3-11.9	9.93-20.57

Bold values are outside the OECD ranges.

Application A549 is for use of high lysine corn in human food. As such, the conventional counterparts used as control should be varieties of maize with a history of safe use commonly used in the same way in human food. "*The data for the non-modified comparator can be the natural ranges published in the literature for commercial varieties or those measured levels in parental or other edible varieties of the species"* (ENV/JM/MONO(2002)25 p.11).

Two reference lines used by the Applicant are considered by the Canadian authorities to be novel food, and were approved for use only 8 years ago[27]. These varieties are Garst 8464 IT and Garst 8590 IT, in the Clearfield production system[28]. They are tolerant to imidazoline-based herbicides.

Five varieties were designated as "high extractable starch corn" (Syngenta N60-N2[29], Golden Harvest H2552[30], DKC 60-15[31], N45-T5[32] and N72-J5[33]). High starch corn is defined as being "lower in fiber than regular corn"[34] and as a corn with normal levels of oil, usually lower protein and with extractable starch yields in the range of 67–72%[35]. High starch corn variety has been grown in the field only since 2001[36]. N45-T5 and N72-J5 are Syngenta seed products branded NK® Brand Extra Edge™ that have been available only since the 2005 growing season[37]. What "experience of establishing safety" has the Authority to justify these reference varieties of corn "based on common use as food" (CAC/GL 44-2003, p. 2 paragraph 8)?

INBI recommendations:

R.26 If the Authority is satisfied with the existing compositional data, we then ask it to indicate how it determined the values provided by the Applicant were as scientifically sound as those used in international guidelines.

R.27 The Authority should evaluate the use of other novel foods as comparators in safety assessments and determine how long a novel food must be used safely before it is considered having a "history of safe use."

Key nutrient and key toxicants comparison

The Applicant has not evaluated all the components that are relevant to the use of LY038 as a human food. "If only agronomical traits are influenced by the genetic modification, derived products need not be analysed separately. In other cases, the additional analysis of derived products can be useful, depending on the nature and purpose of the modification (e.g. deliberately changing the oil composition). This can apply to the following products: maize oil, starch, grits, meal, and flour" (ENV/JM/MONO(2002)25 p.31). The proximate analysis in

[27] http://www.hc-sc.gc.ca/fn-an/gmf-agm/appro/index_e.html .Access date 2 May 2006
[28] http://www.garstseedco.com/GarstClient/PDF/Silage/8464IT.pdf and http://www.garstseedco.com/GarstClient/PDF/Silage/8590IT.pdf .Access date 2 May 2006
[29] http://web.aces.uiuc.edu/value/on-farm/2002%20On-Farm%20VE%20Research %20Report.pdf. Access date 2 May 2006
[30] http://web.aces.uiuc.edu/value/on-farm/2002%20On-Farm%20VE%20Research %20Report.pdf . Access date 2 May 2006
[31] http://www.monsanto.com/monsanto/us_ag/content/enhanced_value/pro_per/pro_per_corn/products.pdf Access date 2 May 2006
[32] http://www.nk-us.com/infosilo/seedguide/seedguide3.asp?slctdEdition=05&slctdState=IA&slctdStateName =Iowa&slctdCounty=19011&slctdCountyName=Benton&slctdCropType=2&slctdVariety=N45-T5 . Access Date 2 May 2006
[33] http://www.nk-us.com/infosilo/seedguide/seedguide3.asp?slctdEdition=05&slctdState=IA&slctdStateName =Iowa&slctdCounty=19013&slctdCountyName=Black%20Hawk&slctdCropType=2&slctdVariety=N72-J5. Access date 2 May 2006
[34] http://www.vegrains.org/english/varieties_highstarch.htm. Access date 2 May 2006
[35] http://www.vegrains.org/documents /2002veg_report/highstarch/hsmktdev. html Access date 2 May 2006
[36] http://www.oardc.ohio-state.edu/hocorn/ip%20faq.htm . Access date 2 May 2006
[37] http://www.plantmanagementnetwork.org/pub/cm/news/2004/extraedge/ .Access date 2 May 2006

maize starch, grits and flour should be provided by the Applicant to prove the equivalence of those derived products for food use.

INBI recommendation:

R.28 The Authority should require the proximate analysis of maize starch, grits and flour derived from LY038.

Saccharopine and α-aminoadipic acid

LY038 has higher levels of the lysine catabolites saccharopine, α-aminoadipic acid and cadaverine. Saccharopine and α-aminoadipic acid levels in particular significantly change the hazard profile of LY038 in cooked and processed foods (as discussed in section 6.1.9 of NZIGE submission 2005). There is 100 times more saccharopine by weight in LY038 than in LY038(-) and at least 50 times more than in the reference controls. The increase in α-aminoadipic acid could not be calculated because it was at levels too low in controls to detect, but averaged 56.69μg/g (39.65 – 82.34 μg/g) in LY038 (DAR, p. 47). So α-aminoadipic acid is at least 10 times higher in LY038 than in conventional corn, based on the Applicant's reported limit of detection (5 ppm or 5 μg/g). While differences in the production of these catabolites may have been anticipated, they are not intended and must be the subject of further studies.

The Authority has argued that "[t]hese analyses…demonstrate a history of exposure to these lysine catabolites from the consumption of commonly available foods." However, these other foods do not have the same concentration of other metabolites as corn, are not eaten or prepared in precisely the same ways as corn, or eaten in the same quantities. For example, based on United States per capita maize consumption (0.52 g/kg BW/day), the mean consumption of saccharopine is estimated to be 290μg/kgBW/day. This is more than 15-times the mean consumption of saccharopine in food containing high levels of saccharopine (e.g. button mushroom at 19μg/kgBW/day). Again, LY038 must be able to substitute for cooked and processed conventional corn and not for button mushrooms, in the human diet.

Saccharopine and α-aminoadipic acid, analogues of standard amino acids, are also probably substrates for Maillard reactions in cooked or processed material. The potential for AGE formation in corn with high saccharopine levels, for example, cannot be inferred from white button mushrooms because LY038 has approximately 20 times the carbohydrate found in mushrooms[38] (81800 vs 4000mg/100g).

Part of the justification for assuming that these catabolites are safe comes from a study of α-aminoadipic acid in lentils (Rozan et al., 2001). Surprisingly, that same study reports that α-aminoadipic acid has neurotoxic activity. The authors were concerned about the levels of this metabolite in lentils, not suggesting that other foods should be introduced with similarly high levels. The Authority should consider not just the absolute amounts in LY038, but the cumulative exposure should LY038 be introduced as the source of corn in a normal diet.

Interestingly, in another study—published by the Applicant—detailing saccharopine and α-

[38] USDA Nutrient Data Laboratory http://www.ars.usda.gov/main/site_main.htm?modecode=12354500. Access date 9 May 2006.

aminoadipic acid levels, they report no significant increase in varieties of maize that have higher lysine levels achieved through a post-transcriptional inhibition of zein translation (Huang et al., 2005). *Only when the maize accumulate higher levels of lysine through the use of recombinant cDHDPS do free lysine and the lysine catabolites increase.* This can be seen in Table 5 (which is a combination of values from Tables 3 and 4 of the Applicant's 2005 publication (Huang et al., 2005)) comparing rows 1-3 with 4-6. Once free lysine is subtracted from the total lysine in high lysine plants, there is no significant difference in lysine levels (Table 5, column 4). Thus, *the use of cDHDPS recombinant plants is a fundamentally different hazard than can be extrapolated from analysis of plants higher in total lysine or other foods higher in total lysine.*

Table 5: Free lysine and not total lysine is key indicator of risk

Mechanism for higher lysine	Total[1,2]				
	Lysine	Free lysine	Lysine-Free lysine	saccharopine	AAA[3]
low zein (PQ15)	2930	64	2866	25	13
low zein (PQ17)	3320	67	3253	13	9
control	2575	43	2529	17	9
cDHDPS (CordapA)	4290	**1838**	2452	**459**	**81**
PQ15 x CordapA	6160	**2908**	3252	**588**	**80**
PQ17 x CordapA	5475	**2498**	2977	**488**	**59**

1. ppm
2. Numbers in bold are significantly higher than control (Table 4 of Huang et al., 2005) before adjusting for free lysine. Reasons for highlighting these are described in text
3. α-aminoadipic acid

INBI recommendations:

R.29 The Authority should justify its conclusion that lysine catabolite levels in a genetically modified variety of corn can be considered safe by comparison to lysine levels in unrelated food sources.

Cadaverine and pipecolic acid

The Authority has not addressed the cadaverine and pipecolic acid levels in LY038. Pipecolic acid levels were approximately double in LY038 in comparison to LY038(-). Since cadaverine is formed by decarboxylation of lysine, the levels also may be significantly higher in LY038. In one section of the DAR, the Authority indicates that cadaverine levels are elevated in LY038 relative to controls (p. 12), but in another section asserts that cadaverine levels could not be measured because they were below detection concentrations in both LY038 and the controls (p. 39). This discrepancy should be addressed.

The Authority also claims that: "FSANZ has not been able to identify any adverse nutritional impacts from increased intakes of these substances in the available scientific literature" (p. 12). According to the US FDA and Department of Human Services of the Victorian Government, Australia, cadaverine is a food hazard. It normally achieves biologically relevant concentrations

when bacteria, particularly those found on fish, "convert free amino acids to biogenic amines (e.g. histamine)" that can cause allergic reactions expressed as mild skin discomfort to nausea, vomiting and diarrhoea[39]. Cadaverine inhibits the enzymes "diamine oxidase (DAO) and histamine-N-methyl transferase (HMT) which convert histamine to harmless degradation products."[40]

Cadaverine may also have other toxic activities. The Victorian Government concluded that while there have been "[n]o limits…set in the Australian Foods Standards Code for putrescine and cadaverine[, t]hese amines may also be toxic in addition to their DAO and HMT enzyme inhibiting effects which potentiate the toxicity of histamine. However, *further research is required to determine the toxicity of all biogenic amines to set safe levels in food for human consumption*" [emphasis ours][40]. Cadaverine could be of particular concern to those taking monoamine oxidase inhibitors (antidepressants). The Authority would also recognize that cadaverine is a potential Maillard reactant as well.

If cadaverine levels are expected to be higher in LY038, then the Authority should assess what levels *in corn* would be relevant to human health concerns, and ensure that LY038 and any hybrids formed with LY038 (particularly other high lysine varieties) do not achieve these levels. If the Authority cannot bind the Applicant to a quantitative ceiling level of cadaverine in all LY038 derivatives, then the Authority should not recommend an amendment to the Food Code.

Pipecolic acid at high levels is found in patients with Zellweger syndrome, and it is "considered to be a neurotransmitter or neuromodulator" because it acts as γ-aminobutyric acid receptor agonist (Fujita et al., 2003). It also "could be involved in the pathogenesis of hepatic encephalopathy" (Fujita et al., 1999). Pipecolic acid is found in two enantiomers, the D- and L-isoforms and both are found in mammals. D- and L-pipecolic acid are formed by enzymatic and non-enzymatic reactions from one another, and they are formed directly from L- and D-lysine. Dietary sources of L-pipecolic acid may provide a source of D-pipecolic acid in humans. "Some studies have reported that heat, alkali or the combination of both treatments [common to food processing] caused amino acid racemization in food proteins", making cooking and processing a likely source of D-pipecolic acid in our food (Fujita et al., 2003).

The Applicant has only provided the Authority with measurements of L-pipecolic acid. Moreover, the Applicant has not measured levels of L- and D-pipecolic acid in cooked and processed LY038 product. Thus, the Applicant has likely under-stated the concentration of pipecolic acid relevant to human health considerations.

It is now clear that D-isomers produced by intestinal bacteria using either D- or L-enantiomers of either pipecolic acid or lysine can be taken up by gut cells. D-pipecolic acid in humans

[39] http://www.cfsan.fda.gov/~dms/3fs3re12.html. http://www.health.vic.gov.au/foodsafety/research/toxins.htm. Access date 21 May 2006.
[40] www.foodsafety.vic.gov.au. Investigation of biogenic amines in fermented fish and fish products. Access date 21 May 2006.

increases when they are on lysine rich diets and decreases if they are taking an antibiotic, further indicating that dietary lysine reaches gut bacteria and is fed back as pipecolic acid (Fujita et al., 1999). It would be impossible to measure the human health effects of elevated levels of pipecolic acid from a compositional study, or using animals with significantly dissimilar intestinal flora in a feeding study, or using raw corn. It is also necessary to assess the effects of high lysine and pipecolic acid with regard to those who suffer from chronic hepatic encephalopathy.

INBI recommendations:

R.30 The Authority should provide quantitative evidence of cadaverine levels in LY038, perhaps by requiring NMR combined with chemometrics and univariant statistics to achieve more sensitive detection. If it does not, then the Authority should require feeding studies using LY038 cooked and processed as normal for human food to assess the potential for cadaverine at elevated levels in corn to produce food hazards.

R.31 The Authority should assess the sensitivity of those on monoamine oxidase inhibitors to measured levels of cadaverine in a diet composed of LY038 corn.

R.32 The Authority should report total pipecolic acid levels in LY038 and not just L-pipecolic acid levels.

R.33 The Authority should assess the contribution the intestinal flora will make to pipecolic acid levels in consumers who eat corn with high levels of lysine, free lysine and pipecolic acid.

R.34 The Authority should explain how it has considered the impact of pipecolic acid in high lysine corn on those suffering from chronic hepatic encephalopathy.

Measurements of free and total amino acids

The Applicant reports amino acid levels as a proportion of amino acids. This can mask important changes in the amounts of amino acids, especially in distributions between protein-incorporated and free amino acids (Toro et al., 2003).

INBI recommendation:

R.35 The Applicant has reported absolute amounts (by weight) of the amino acids in its most recent study (MSL-18881) but the Authority has accepted the statistical analysis based on %AA. The Authority should present the statistical analysis based on absolute amounts by weight.

Lysine synthesis and lysine catabolism (section 2.2.3-2.2.7 in NZIGE submission)

Animals feeding on plants have adapted to particular levels of total and free lysine and any derivative metabolites. 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.

The Applicant has recently published a paper revealing the analysis of protein distribution in varieties of corn hybridized with a parent of highly similar if not identical description to LY038 (Huang et al., 2005). This description raises significant discrepancies with the application.

The Applicant reported to the Authority that "[t]herefore, in Lysine maize LY038, the expected total lysine would range from 3500 to 5300 ppm" (p. 2). In Huang *et al.* (2005), hybrids were expressing up to 6160 ppm total lysine, an increase of up to 100% over the levels reported in the Applicant's controls in A549.

The Applicant reported to the Authority that "[l]evels of free lysine are expected to be in the range of 1000 to 2500 ppm in Lysine maize LY038 grain" (p. 2). In Huang *et al.* (2005), hybrids were expressing nearly 3000 ppm.

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 aminoadipic 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, Toro et al., 2003). In Huang *et al.* (2005), the Applicant has found cDHDPS recombinant plants have the same LOR-SDH activities as plants that do not accumulate lysine. However, in mutants with high lysine due to changes in zein protein increases, the activity of this pathway is increased. Combined, the studies of Azevedo's group and Huang *et al.* (2005) confirm that cDHDPS recombinants achieve their levels of lysine accumulation in part by compensatory changes in lysine catabolism (either decreased activity or failing to increase activity relative to conventional varieties), a factor that cannot be predicted in advance for novel hybrids.

For whatever reason, cDHDPS has achieved a higher concentration of lysine, especially free lysine, in seed in line LY038 (MSL-19172). In conventional maize mutants with higher lysine yields, this has been due to an increase in protein incorporated lysine rather than free lysine (Toro et al., 2003).

INBI recommendations:

R.36 The Authority should provide evidence that hybrids with the LY038 event have the same absolute amounts of glutamate, free lysine, saccharopine and α-aminoadipic acid as LY038 to assure the Authority that LY038 has no physiological behaviours that are unique to its genetic background with regard to lysine catabolism in seed.

R.37 The Authority should address the difference in expected ranges of total and free lysine (as reported in A549) and the higher values already known to exist in hybrids created by the Applicant by explaining how it has determined what absolute levels of these compounds in corn could be a cause for concern.

There are indicators that elevated levels of cadaverine in corn could have physiological consequences altering other nutrient levels or in the creation of potential food hazards. Cadaverine levels were found to follow heat stress in marigolds, indicating that cadaverine has a role in recovery from stress. Chronic high levels of cadaverine, therefore, are not the norm for plants. According to the authors of the study "the superproduction of cadaverine would result in SAM [S-adenosyl-L-methionine] deficiency and, consequently, in the suppression of spermidine and spermine syntheses. In addition, in some cadaverine-containing plants, an unconventional behavior of cadaverine relative to ethylene and putrescine-type polyamines was observed" and "[t]he data obtained support the suggestion that cadaverine serves as a stress signal at the whole-plant level" (Shevyakova et al., 2000). LY038 may have the physiology of perpetually stressed plants and not conventional corn.

INBI recommendation:

R.38 The Authority should provide evidence that LY038 and any hybrids with the LY038 event have the same absolute amounts of SAM and spermidine, and report on feeding studies using LY038 corn prepared as per normal for human consumption to assure the Authority that LY038 has no physiological behaviours that are unique to its genetic background with regard to lysine catabolism in seed.

Characterisation of novel protein

Our recommendations are based on the evaluation of the studies MSL-18585 "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" written by Rice, E.A., Kapadia, S.A., Thoma, R.S. and Hileman, R.E. (Monsanto company 2003) and MSL-18365 "Characterisation of the *E. coli*-Produced *Corynebacterium glutamicum* Dihydrodipicolinate Synthase (cDHDPS) Protein" written by Rice, E.A., Kapadia, S.A., Dalton, C.M., Brown, T.P., Thoma. R.S., Hileman, R.E. and Astwook, J.D. (Monsanto Company 2003).

Ambiguities in protein identification

The Authority has concluded that "SDS-PAGE and Western blotting techniques were used to demonstrate that the cDHDPS protein expressed in LY038 corn was of the expected size. N-terminal sequencing and MALDI-TOF mass spectrometry further confirmed that the desired protein was expressed in LY038. Glycosylation analysis showed that cDHDPS is not glycosylated in LY038" (DAR, p. 21). However, this conclusion does not seem to be consistent with experimental fact.

(1) In Table 3 of Appendix MSL-18585 the Applicant makes clear that proteins of 33, 34 and 35kDa band are detected using SDS PAGE. The proteins of 35kDa are present at a relative abundance of less than 10%, the arbitrary cut off imposed by the Applicant (see "Methods, Specific Analyses to be Performed" p. 43 MSL-18585).

(2) Only the 33kDa bands produced in *E. coli* and *in-planta* are subsequently used for N-