FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

APPLICATION A1018 – FOOD DERIVED FROM HIGH OLEIC ACID SOYBEAN LINE DP-305423-1

SUPPORTING DOCUMENT 1

Safety Assessment Report

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SUMMARY AND CONCLUSIONS

Background

A new variety of genetically modified soybean (*Glycine max*), known as soybean line DP-305423-1 (hereafter referred to as soybean 305423), has been generated which contains an increased level of oleic acid in the seed. The increased level of oleic acid has been achieved by decreasing the expression of an endogenous soybean fatty acid desaturase gene (*gm-fad2-1*) using gene silencing. The genetic modification also results in a decreased level of linoleic acid.

Soybean 305423 also contains a modified version of a soybean acetolactate synthase (*als*) gene (*gm-hra*). The GM-HRA enzyme can function in the presence of the ALS-inhibiting class of herbicides, thereby conferring a degree of tolerance to those herbicides.

The Applicant has not applied to the Office of the Gene Technology Regulator, or the Environmental Risk Management Authority in New Zealand for a licence to grow soybean 305423. Therefore foods derived from soybean 305423, if approved by FSANZ, would only enter the food supply in Australia and New Zealand through imported products unless a commercial growing licence were to be issued at some later date.

History of Use

Soybean (*Glycine max* (L.) Merr.) is grown as a commercial crop in over 35 countries worldwide. Soybean-derived products have a range of food and feed as well as industrial uses and have a long history of safe use for both humans and livestock. Oil accounts for 94% of the soybean products consumed by humans and is used mainly as a salad and cooking oil, bakery shortening, and frying fat as well as being incorporated into processed products such as margarine.The major food product to be derived from the high oleic acid soybean line would be oil that is predominantly used in spraying and frying applications and might replace heat stable fats and oils such as hydrogenated soybean and rapeseed oil or palm olein/vegetable oil blends.

Molecular Characterisation

Soybean 305423 contains two introduced coding sequences and associated regulatory elements. The first is a partial copy of a soybean fatty acid desaturase gene (*gm-fad2-1*) corresponding to approximately 40% of the middle region of the open reading frame of the endogenous gene. Molecular analysis indicates that, as intended, transcription of this partial sequence results in suppression of expression ('silencing') of the endogenous *gm-fad2-1* gene.

The second coding sequence introduced into soybean 305423 is the *gm-hra* gene encoding a modified version of the soybean acetolactate synthase (*als)* enzyme (GM-HRA) that confers a degree of tolerance to acetolactate-inhibiting herbicides. The *gm-hra* gene was used as a selectable marker to identify genetically modified plants during their initial development in the laboratory. Commercial levels of herbicide tolerance have not been conferred on soybean 305423. There are no antibiotic-resistance markers in soybean 305423.

Comprehensive molecular analyses of soybean 305423 indicate that there are four insertion sites at a single genetic locus. These sites contain multiple copies, both intact and truncated, of the *gm-fad2-1* partial sequence and a single copy of the *gm-hra* gene. Breeding over three generations has confirmed stability of the introduced genetic elements

and segregation data indicate their Mendelian inheritance. Two unexpected ORFs are present at junctions associated with two of the insertion sites; neither of these ORFs is likely to be expressed due to the absence of the necessary regulatory sequences.

Characterisation of Novel Protein

The introduced *gm-fad2-1* element is a partial sequence rather than a complete gene, therefore no novel protein is produced as a consequence of its transcription in cells of the genetically modified (GM) soybean.

Soybean 305423 therefore expresses only one novel protein, GM-HRA. This protein is 656 amino acids in length, has a predicted molecular weight of 71 kDa and differs from the endogenous soybean ALS by two amino acids at positions 183 and 560. The amino acid changes were generated by site-specific mutagenesis.

Expression analysis of the HRA protein has shown that it is expressed in leaves, roots, seeds and forage (above ground parts, including immature pods, that are harvested for use as feed for livestock). The average content in mature seeds is 2.5 ng/mg dry weight (range 0 – 4.9 ng/mg).

Extensive studies have been done to confirm the identity and physicochemical and functional properties of the expressed GM-HRA protein, as well as to determine its potential toxicity and allergenicity. The protein conforms in size and amino acid sequence to that expected, does not exhibit any post-translational modification including glycosylation, and demonstrates the predicted enzymatic activity.

Bioinformatic studies with the GM-HRA protein have confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that the protein would be rapidly degraded following ingestion, similar to other dietary proteins. Furthermore, the GM-HRA protein is heat labile. An acute oral toxicity study in mice with the GM-HRA protein have also confirmed the absence of toxicity. Taken together, the evidence indicates that GM-HRA protein is neither toxic nor likely to be allergenic in humans.

Compositional Analyses

Detailed compositional analyses were done to establish the nutritional adequacy of seed-derived products from soybean 305423 and to characterise the intended as well as any unintended compositional changes. Analyses were done of proximate (crude fat/protein, fibre, ash), amino acid, fatty acid, vitamin, mineral, sucrose, isoflavone, and anti-nutrient content. The levels were compared to levels in the non-GM parent and to those in four non-GM commercial soybean cultivars. These analyses indicated that the seeds of high oleic acid soybeans are significantly changed from those of the parental line with respect to their fatty acid profile.

The mean oleic acid content has been increased from 21.1% in the parental soybean to 76.5% in the high oleic soybean line, the linoleic acid and linolenic acid contents have been concomitantly decreased from a mean level of 52.5% to a mean level of 3.62% for linoleic acid and from 9.35% to 5.39% for linolenic acid. The level of linolenic acid in soybean 305423, while significantly lower than that in the control is, nonetheless within the normal range found in soybeans while the levels of oleic acid and linoleic acid in soybean 305423 are outside the normal range. The level of oleic acid in soybean 305423 oil is comparable to that in a range of other commercially available and commonly consumed vegetable oils. Consumption of such levels of oleic acid does not pose any safety concerns.

There has also been an unintended increase in two minor fatty acids, heptadecanoic acid and heptadecenoic acid. Together, these two fatty acids constitute approximately 2% of the total fatty acid content in soybean 305423, compared to approximately 0.17% in the control line. Both fatty acids are consumed as part of a normal human diet and are readily metabolised therefore the small increase in their levels is not considered to pose any safety concerns.

In terms of other analytes, seeds of soybean 305423 were found to be compositionally equivalent to those from the non-GM parent and other non-GM commercial soybean cultivars. Several minor differences in key nutrients and other constituents were found however, the mean levels observed are within the range of values observed for the non-transgenic comparator and within the range of natural variation.

In addition, no difference between soybean 305423 and the nontransgenic parent were found in allergenicity studies using sera from soybean-allergic individuals.

With the exception of oleic acid, linoleic acid, heptadecanoic acid and heptadecenoic acid, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in seed from soybean 305423 when compared with conventional soybean cultivars currently on the market.

Nutritional Impact

Dietary exposure assessments of the fatty acids contained in soybean were done in the U.S., as well as in Australia and New Zealand, to determine the human nutritional impact of the compositional changes to the fatty acid profile of the soybean. These assessments indicate that the substitution of soybean oil with oil from soybean 305423 would have minimal effect on the intake of dietary significant fatty acids. At most, if soybean oil was replaced with the oil from soybean 305423, there may be a marginal increase (up to 6%) in intake of oleic acid and a marginal decrease (up to 10%) in linoleic acid intake. In terms of both cooking quality and nutrition, the replacement of linoleic acid by oleic acid means that partial hydrogenation is not required to stabilise the fatty acids. This in turn, has the potential to reduce the intake of undesirable *trans* fats in the diet. It is significant to note that, while the levels of oleic and linoleic acids have been altered, the total percentage of unsaturated fatty acids is approximately the same in the control and in soybean 305423. Taken overall, it is concluded that use of oil from soybean 305423 would have minimal nutritional impact. This conclusion is consistent with that reached by FSANZ for a previous high oleic acid soybean application.

Two animal feeding studies, in chickens and rats, indicate that the high oleic acid soybeans are nutritionally adequate and equivalent to non-GM soybeans in their ability to support typical growth and well-being.

Conclusion

No potential public health and safety concerns have been identified in the assessment of high oleic acid soybean 305423. On the basis of the data provided in the present Application, and other available information, food derived from soybean 305423 is considered as safe for human consumption as food derived from conventional soybean cultivars.

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LIST OF ABBREVIATIONS

ACP	acyl carrier protein
ADF	acid detergent fibre
AI	adequate intake
AMDR	acceptable macronutrient distribution range
ALS	acetolactate synthase
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
C17:0	heptadecanoic acid
C17:1	heptadecenoic acid
C18:0	stearic acid
C18:1	oleic acid
C18:2	linoleic acid
C18:3	linolenic acid
DNA	deoxyribonucleic acid
dw	dry weight
ELISA	enzyme linked immunosorbent assay
FAD	fatty acid desaturase
FAO	Food and Agriculture Organization of the United Nations
FARRP	Food Allergy Research and Resource Program
FRT	flippase (FLP) recognition target
FSANZ	Food Standards Australia New Zealand
GC	gas chromatography
GM	genetically modified
GM-FAD2-1	fatty acid desaturase from *Glycine max*
GM-HRA	herbicide resistant ALS from *Glycine max*
hyg	hygromycin resistance gene
IgE	immunoglobulin E
ILSI	International Life Sciences Institute
kDa	kilo Dalton
KTi3	*Kunitz trypsin inhibitor gene 3*
LLOQ	lower limit of quantitation
LSM	least squares mean
MALDI-MS	matrix assisted laser desorption ionization mass spectrometry
NHANES	National Health and Nutrition Examination Survey (U.S.)
NH&MRC	National Health & Medical Research Council (Australia)
NCBI	National Center for Biotechnology Information
NDF	neutral detergent fibre
NUTTAB	Nutrient Tables (Australian Food Composition Tables)
OECD	Organisation for Economic Co-operation and Development
ORF	open reading frame
ori	origin of replication
PCR	polymerase chain reaction
RNA	ribonucleic acid
dsRNA	double-stranded RNA
mRNA	messenger RNA
sams	*S-adenosyl-L-methionine synthetase* gene
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
TAG	triacylglycerol
TCA	tricarboxylic acid
U.S.	United States of America
UTR	untranslated region
WHO	World Health Organisation

1. INTRODUCTION

Soybean line DP-305423-1 (hereafter referred to as soybean 305423) has been genetically modified (GM) to produce seeds with an increased level of oleic acid. This modification results in concomitant decreases in the level of linoleic acid. Seeds of this high oleic acid soybean line are intended for use in the production of PLENISH™[1] high oleic acid soybean oil in an identity preserved production system. Many conventional cooking oils, including soybean, have poor oxidative stability due to naturally high levels of linoleic acid (Smith *et al*., 2006) and thus require additional processing, such as chemical hydrogenation to stabilise them. This, in turn, leads to the undesirable formation of *trans* fats. The high oleic acid oil derived from soybean 305423 provides an alternative to partially hydrogenated oils. It would be used primarily to replace commercial frying oils used with french fries, potato chips, tortilla chips and other corn based snacks, commercially fried fish, and commercially fried chicken as well as oils used as a spray treatment on crackers, pretzels, and other grain snacks.

Recently there has been interest in genetically modifying oilseed fatty acids, particularly via directed gene down-regulation strategies (see discussion in Durrett *et al*., 2008; Liu *et al*., 2002). The increased levels of oleic acid in soybean 305423 have been achieved through the introduction of a partial gene sequence (fragment) designed to decrease the expression of one of the endogenous soybean fatty acid desaturase genes (*gm-fad2-1*[2] that encodes microsomal ω-6 desaturase (Heppard *et al*., 1996). The endogenous *gm-fad 2-1* gene plays a major role in desaturation of storage lipids synthesised during the mid-maturation stages of seed development. More specifically, it is responsible for the conversion of oleic acid (18:1) to linoleic acid (18:2) (Heppard *et al*., 1996), which is the major unsaturated fatty acid present in soybean oil. The decrease in endogenous expression is achieved through a mechanism known as co-suppression (see Section 3.2). The presence of the introduced fragment of the fatty acid desaturase gene results in suppression of expression of the endogenous fatty acid desaturase gene, thus significantly reducing linoleic acid formation and leading to the accumulation of oleic acid. The high levels of oleic acid are confined largely to the seed, as this is primarily where the *gm-fad2-1* gene is expressed (see Section 3.2). Fatty acid biosynthesis functions normally in other plant parts such as the leaves.

Soybean 305423 also contains a complete gene sequence that encodes a modified version of the soybean acetolactate synthase (ALS)[3] protein (Falco and Li, 2003) which confers a degree of tolerance to ALS-inhibiting herbicides (see Section 4.2). The gene is known as *gm-hra*. In soybean 305423 it is used as a marker gene in order to select potential transformants at the early stage of the transformation procedure and is not intended for agronomic use. No antibiotic-resistance marker genes are present in soybean 305423.

2. HISTORY OF USE

2.1 Host and Donor Organism

The host organism is soybean (*Glycine max* (L.) Merr.) and both of the introduced coding sequences have been derived from gene sequences normally present in non-GM soybean. The other genetic elements used in the gene constructs have also largely been derived from soybean.

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[1] PLENISH™ is a trademark of Pioneer Hi-Bred International, Inc. In the original Application, it was indicated that the trademark name would be TREUS™; the Applicant subsequently notified FSANZ in August 2009 of the change.
[2] The designation 'gm' in *gm-fad2-1* and *gm-hra* stands for *Glycine max*, the Latin name for soybean.
[3] The nomenclature for the enzyme is confused in the literature and the term acetohydroxyacid synthase (AHAS) is sometimes used synonymously with ALS. While AHAS more correctly applies to the enzyme referred to in this safety assessment (Duggleby and Pang, 2000), ALS is used for consistency with early literature.

Soybean is grown as a commercial crop in over 35 countries worldwide (OECD 2000) and has a long history of safe use amongst both humans and livestock. The major producers of soybeans, accounting for 90% of world production, are the U.S., Argentina, Brazil and China. In 2007, soybeans represented 56 percent of total world oilseed production, and 32 percent of those soybeans were produced in the U.S. where they provided 71 percent of the edible consumption of fats and oils (The American Soybean Association, 2008). Australia, while a net importer of soybean, grows crops in latitudes extending from the tropics (16° S) to temperate regions (37° S), mainly in the eastern states and as a rotational crop (James and Rose, 2004). The seed is used mainly to produce meal for use in animal feed (Grey, 2006).

In many soybean producing countries, GM soybean (mainly with a herbicide tolerant trait) accounts for a significant proportion of the total soybean grown eg U.S. (91%); Argentina (99%); Brazil (63%); South Africa (87%); Uruguay (99%) (Brookes and Barfoot, 2009).

Unprocessed (raw) soybeans are not suitable for food use, and have only limited feed uses, as they contain toxicants and anti-nutritional factors, such as lectins and trypsin inhibitors (OECD, 2001a). Appropriate heat processing inactivates these compounds. Soybean products are derived either from whole or cracked soybeans:

- whole soybeans are used to produce soy sprouts, baked soybeans, roasted soybeans and traditional soy foods such as miso, tofu, soy milk and soy sauce.
- cracked soybeans have the hull (seed coat) removed and are then rolled into flakes which undergo solvent extraction to remove the oil. This crude oil is further refined to produce cooking oil, shortening and lecithins as well as being incorporated into a variety of edible and technical/industrial products. The flakes are dried and undergo further processing to form products such as meal (for use in eg livestock, pet and poultry food), protein concentrate and isolate (for use in both edible and technical/industrial products), and textured flour (for edible uses). The hulls are used in mill feed.

Oil from conventional soybeans has five major fatty acid components – palmitic acid (16:0 – 12% of total fatty acid content), stearic acid (18:0 – 4%), oleic acid (18:1 – 23%), linoleic acid (18:2 – 53%) and linolenic acid (18:3 – 8%) (Lee *et al.*, 2007).

The soybean cultivar 'Jack' has been used as the parental variety for the high oleic acid trait described in this application, and thus is regarded as the near-isogenic line for the purposes of comparative assessment. 'Jack' was released in the U.S. in August 1989 for its resistance to soybean cyst nematode and higher yield when compared to cultivars of similar maturity (Nickell *et al.*, 1990).

3. MOLECULAR CHARACTERISATION

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects: the transformation method together with a detailed description of the DNA sequences introduced to the host genome; a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation; and the genetic stability of the inserted DNA and any accompanying expressed traits.

Studies submitted:

Weber, N.; Dietrich, N. (2007). Characterization of Soybean Event DP-305423-1: Detailed Physical Map of Insert Region by Southern Analysis. Pioneer Hi-Bred International, Inc. Study ID: PHI-2005-045 (unpublished).

Weber, N. & Dietrich, N. (2007) Characterization of DP-305423-1 Soybean: Genetic Stability over Two Generations and Backbone DNA Analysis. Pioneer Hi-Bred International, Inc. Study ID: PHI-2005-069 (unpublished).

Brink, K.; Dietrich, N. (2007). Characterization of Soybean Event DP–305423-1: Genetic Equivalence of the Inserted DNA within a Single Generation. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-027 (unpublished).

Weber, N.;Brink, K.;Dietrich, N. (2007). Molecular Characterization of DP-305423-1 Soybean. Pioneer Hi-Bred International, Inc. Study ID: PHI-2007-100 (unpublished).

Chui, C.-F.; Tyree, C.W.;Zhong, C. (2006). Gene Expression Studies on DP-305423-1 by Northern Blot Analysis on Leaf Tissue and developing Seed. Pioneer Hi-Bred International, Inc. Study ID: PHI-2005-046 (unpublished).

Stecca, K.; Meyer, K.; Henderson, N.; Cressman, R.; Hunt, S.; Young, J.; Barden, K.;Zhong, C. (2007). Sequence Characterization of Inserts and Genomic Border Regions of Soybean Event DP-305423-1. Pioneer Hi-Bred International, Inc. Report ID: PHI-2006-010/040 (unpublished).

In addition to information in the unpublished studies submitted, considerable detail about the generation and characterisation of soybean 305423 can also be found in Kinney et al. (2008).

3.1 Method used in the genetic modification

Soybean cultivar ‘Jack’ was transformed with the linear DNA fragments designated PHP19340A (Figure 1) and PHP17752A (Figure 2) using a biolistic method (Klein *et al.*, 1987). Fragment PHP19340A (2,924 bp) contains the *gm-fad2-1* partial sequence, while fragment PHP17752A (4,512 bp) contains the *gm-hra* gene. Both fragments were derived by linearisation of a circular plasmid and have identical plasmid backbones.

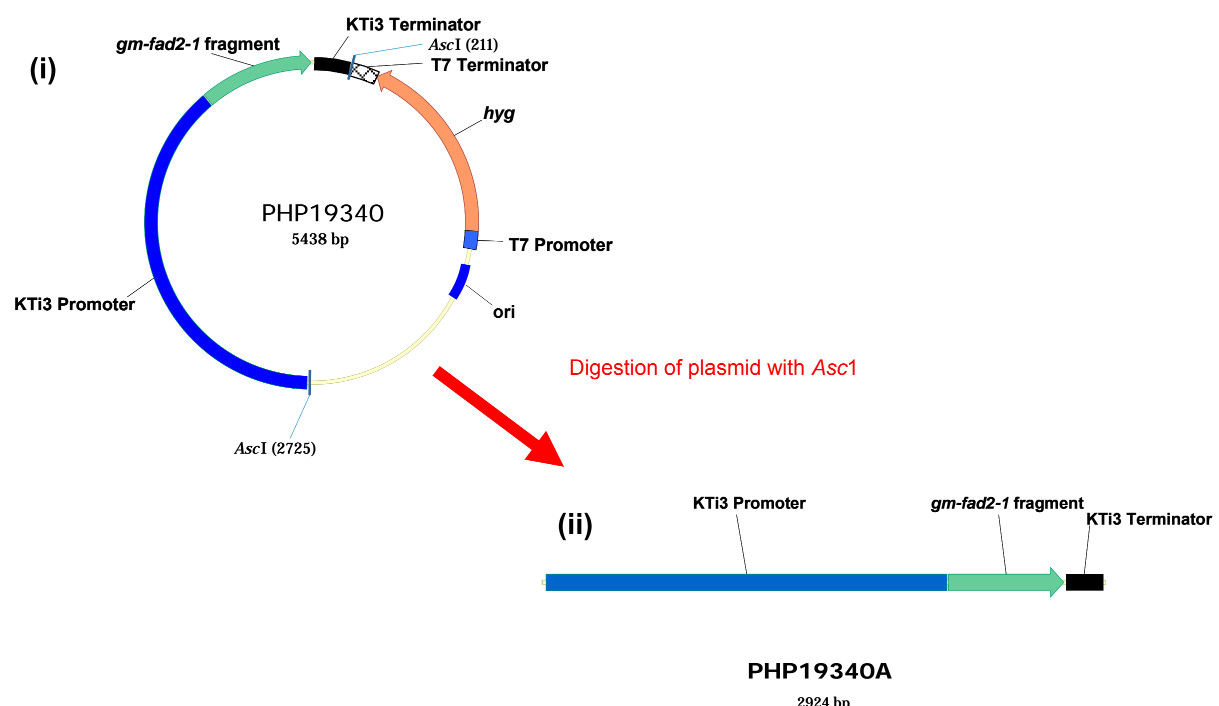

Figure 1: Genes and regulatory elements contained in plasmid PHP19340 (i) and the linear fragment PHP19340A (ii) derived from it.

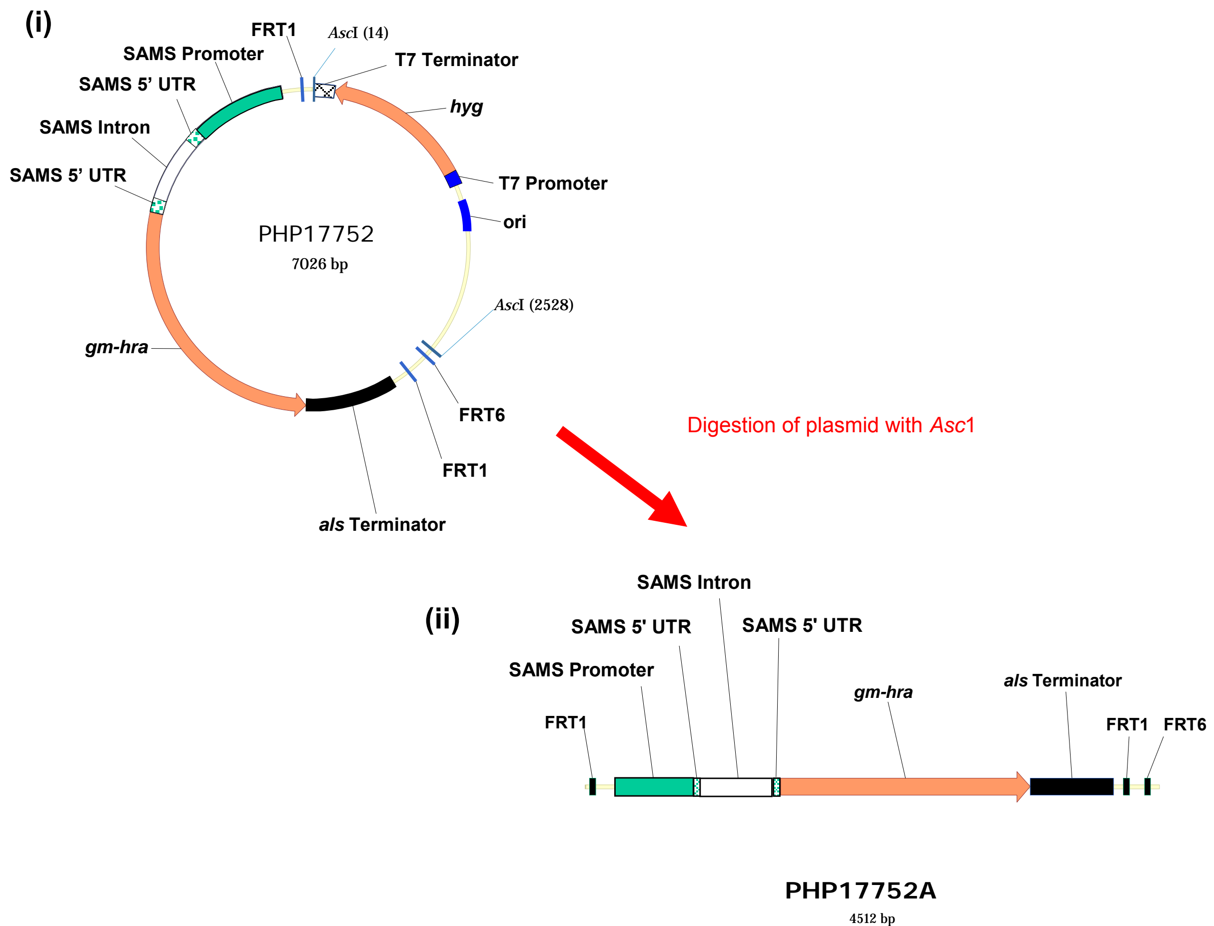

Figure 2: Genes and regulatory elements contained in plasmid PHP17752 (i) and the linear fragment PHP1772A (ii) derived from it.

Details of the method of genetic modification are given in Kinney et al. (2008). In summary, cells from an embryogenic suspension culture were bombarded with gold particles coated with a mixture of the two purified fragments. After 7 days, the cells were placed in a medium containing chlorsulfuron to select putative transformants. Tissue from plants that regenerated was analysed by Southern blot hybridisation to confirm the presence of the introduced genes. Further evaluation of confirmed GM plants was undertaken to select those plants/events with a superior fatty acid profile coupled with good agronomic performance. Backcrossing and further evaluation eventually led to selection of line 305423 for commercial consideration (see Figure 3).

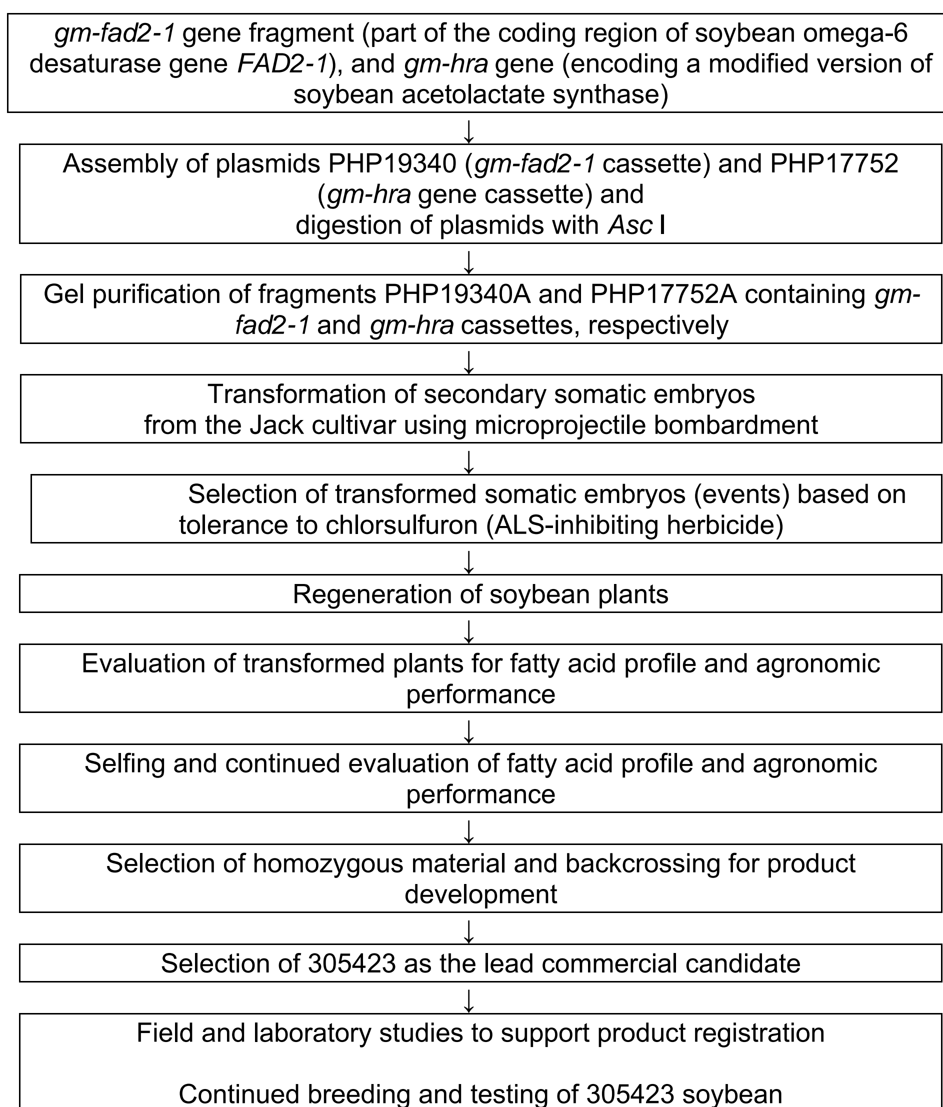

Figure 3: Summary of transformation and evaluation procedures leading to selection of soybean 305423.

3.2 Breeding of soybean 305423

Figure 3 summarises how a single T0 plant of soybean 305423 was produced by genetic modification of cultivar 'Jack'. Following its selection, a breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the molecular and genetic characteristics of the line, and
- ensuring that the 305423 germplasm is incorporated into a wide cultivar base for commercialisation of GM high oleic acid soybeans.

This breeding programme is summarised in Figure 4. The T0 plant was selfed and a single plant (T1) was selected from the progeny. Subsequent rounds of selfing and seed bulking then followed to produce the 'T' generations. Plants from the T3 generation were crossed with a number of Pioneer elite cultivars to produce an F1 generation which was either selfed to produce F2 and F3 generations, or backcrossed to the appropriate parental elite cultivar. Null segregant plants are those plants developed from the transformation procedure that do not contain either of the introduced genes. They are useful as controls in various

evaluations (see Sections 4.3, 5 & 6).

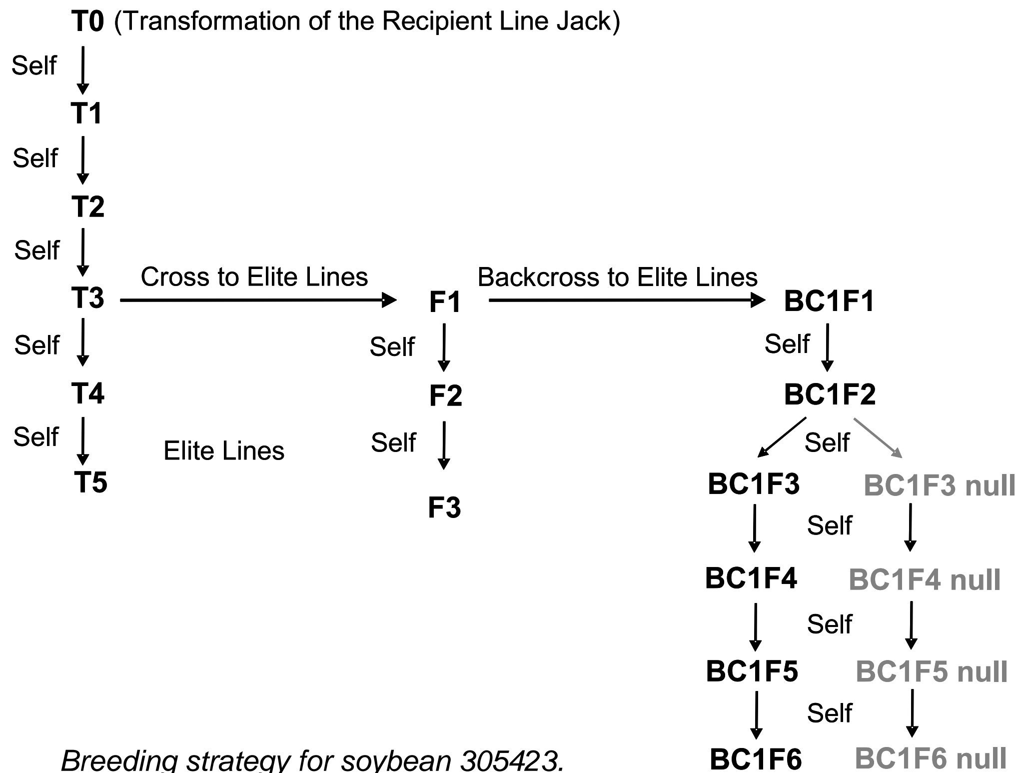

Figure 4. Breeding strategy for soybean 305423.

3.3 Function and regulation of introduced gene sequences

Information on the coding sequences, nucleotide sequences and regulatory elements in the two linear DNA fragments PHP19340A and PHP17752A is given in Table 1.

Table 1: Description of the genetic elements contained in the introduced linear DNA fragments .

Linear fragment	bp location on linear fragment	Size (bp)	Genetic element	Source	Function
PP19340A (2,924 bp)	1 - 18	18	polylinker region[a]		
	19 - 2102	2084	*Kti3* promoter	soybean	Promoter region from *Kunitz trypsin inhibitor gene 3* (*Kti3*) – drives expression in maturing seeds.
	2103 - 2113	11	polylinker region		
	2114 - 2710	*597*	*gm-fad2-1* partial gene sequence	soybean	Coding sequence – contains 40% of the ORF of the endogenous soybean ω-6 desaturase gene – silences the endogenous gene
	2711 - 2720	10	polylinker region		
	2721 - 2916	196	*Kti3* terminator	soybean	Terminator region from soybean *Kunitz trypsin inhibitor gene 3* (*Kti3*) – required for termination of transcription of the coding sequence.
	2916 - 2924	8	polylinker region		
PP17752A (4,512 bp)	1 - 25	25	polylinker region		
	26 - 76	51	FRT1	*Saccharomyces cerevisiae*	FLP recombinase recombination site
	77 - 222	146	polylinker region		
	223 - 867	645	*sams* promoter	soybean	Constitutive promoter from *S-adenosyl-L-methionine synthetase* (*sams)* gene
	868 - 926	59	*sams* 5'-UTR	soybean	5' UTR of *sams* gene
	927 - 1517	591	*sams* intron	soybean	Intron within the 5' UTR of *sams* gene
	1518 - 1533	16	*sams* 5'-UTR	soybean	5' UTR of *sams* gene
	1534 - 3504	1971	*gm-hra*	soybean	Coding sequence - modified version of *als* gene – confers tolerance to sulfonylurea herbicides
	3505 - 4156	652	*als* terminator	soybean	Terminator from *als* gene – required for termination of transcription of the coding sequence
	4157 - 4231	75	*polylinker region*		
	4232 - 4282	51	FRT1	*Saccharomyces cerevisiae*	FLP recombinase recombination site
	4283 - 4396	114	*polylinker region*		
	4397 - 4447	51	FRT6	*Saccharomyces cerevisiae*	FLP recombinase recombination site
	4448 - 4512	65	*polylinker region*		

[a] polylinker regions are required for cloning genetic elements.

3.3.1 gm-fad2-1 *partial gene sequence*

Plant oils are primarily composed of triacylglycerols (TAGs) that in turn comprise 3 fatty acid chains usually 16 or 18 carbons long (Durrett *et al.*, 2008). The synthesis of these fatty acids in plants occurs in plastids and essentially results in the formation of 16:0, 18:0 and 18:1 fatty acids; odd chained and other even chained fatty acids are produced to a lesser degree – see Section 5.3. These fatty acids are esterified to acyl carrier protein (ACP) – see Figure 5. Thioesterases then release them from ACP so that they may be exported to the endoplasmic reticulum for desaturation (addition of double bonds) by fatty acid desaturases (FADs) and assembly into TAGs. FADs are also found in most animals and in some eubacteria.

The synthesis of polyunsaturated fatty acids in developing oilseeds is catalysed by two membrane-associated FADs that sequentially add a second and third double bond to oleic acid (Kinney, 1999). The second double bond, converting oleic acid (18:1) to linoleic acid (18:2), is added at the ω-6 position by ω-6 (δ-12) desaturase, encoded by the *fad2* gene (Heppard *et al*., 1996; Okuley *et al*., 1994). The third double bond, converting linoleic acid to linolenic acid (18:3), is added at the ω-3 (δ-15) position by an ω-3 desaturase, encoded by the *fad3* gene (Yadav *et al*., 1993).

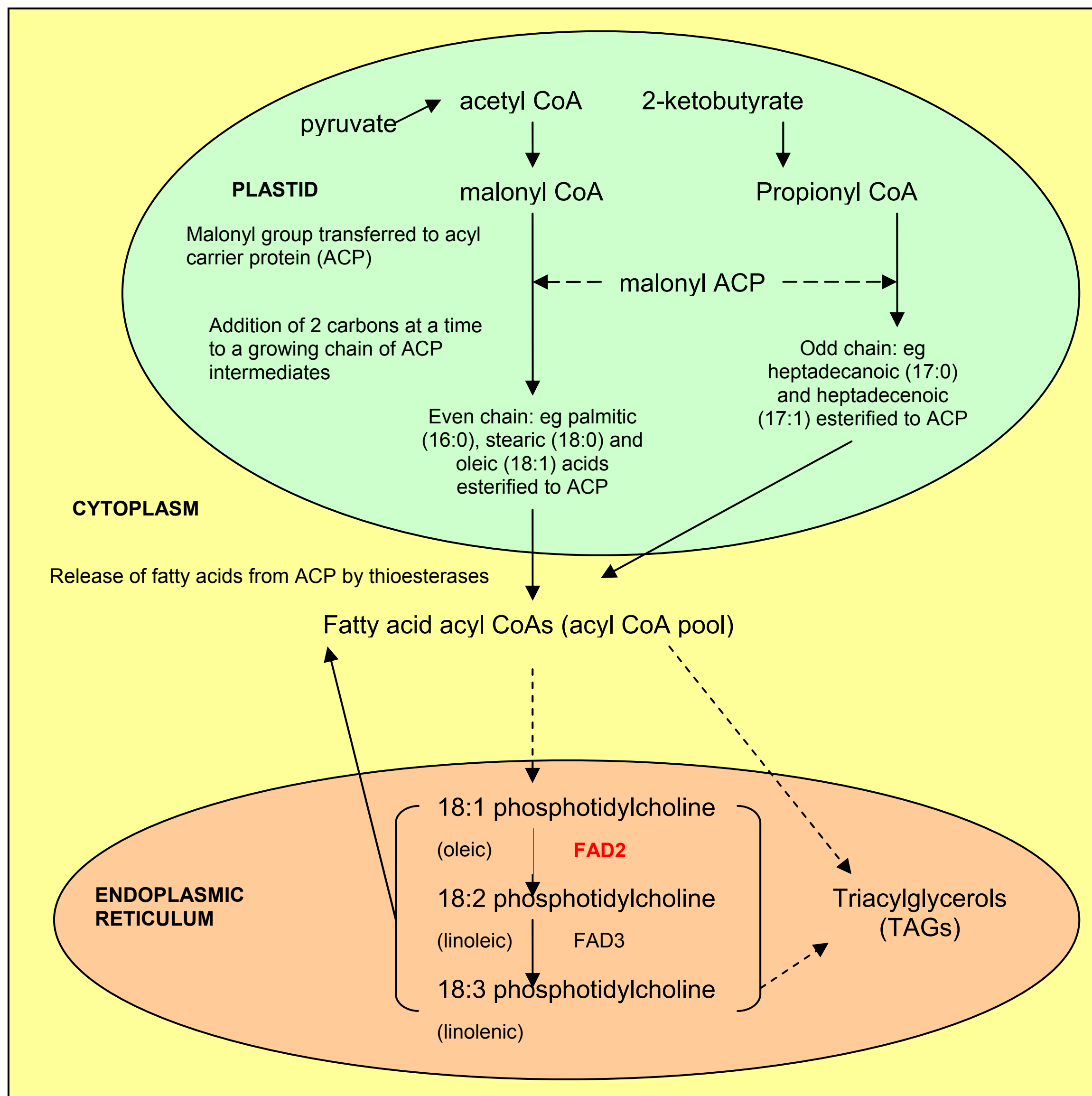

Figure 5: A simplified schematic summary of the synthesis of fatty acids in plants (adapted from Kinney (1999) and Durrett et al. (2008)).

In soybean, there are two *fad2* genes (*fad2-1* and *fad2-2*). The *fad2-1* gene, used as the target for genetic modification in soybean 305423, is expressed more strongly in the developing seed and increases during the period of oil deposition, starting around 19 days after flowering (Heppard *et al*., 1996). Its expression is responsible for the synthesis of the polyunsaturated fatty acids found in the oil fraction. The *fad2-2* gene is expressed in the seed, leaf, root and stem at a constant level and its gene product is responsible for the synthesis of the polyunsaturated fatty acids present in cell membranes. The two genes *fad2-1* and *fad2-2* share approximately 70% homology in the coding region (data supplied in

Pioneer Hi-Bred International, Inc. Study ID: PHI-2005-046).

The *gm-fad2-1* partial sequence introduced into soybean 305423 is 597 bp in length and is driven by the promoter region from the *Kunitz trypsin inhibitor gene 3* (*Kti3*), also from soybean (Jofuku and Goldberg, 1989). This promoter drives a high transcription rate in the developing embryo during the mid-maturation stage of seed development; and is much less active in other parts of the plant. At the 3' untranslated region of the introduced fragment is an mRNA termination region also derived from the soybean *KTi3* gene.

The purpose of introducing the *gm-fad2-1* partial sequence in soybean 305423 is to bring about post-transcriptional 'silencing'[4] of the endogenous *gm-fad2-1* gene so that the fatty acid biosynthesis pathway is effectively blocked at the stage where oleic acid is converted to linoleic acid. Post-transcriptional gene silencing can be a naturally occurring phenomenon in plants and is particularly associated with defence against viral attack (see eg Baulcombe, 2004; Waterhouse *et al.*, 2001). The term encompasses a range of RNA silencing pathways (eg RNA interference, co-suppresion) that seem to be triggered by the presence in the plant of complementary RNA strands that form double-stranded RNA (dsRNA). This dsRNA is recognised by the plant as 'undesirable' and an enzyme known as Dicer is activated and cleaves the dsRNA into short nucleotide sequences. These sequences, in turn, become guides for other enzymes that destroy complementary RNA sequences. In the context of soybean 305423, the *gm-fad2-1* partial sequence corresponds to approximately 40% of the middle region of the open reading frame of the endogenous *gm-fad2-1* gene. It The Applicant speculates that suppression of expression of the endogenous *gm-fad2-1* gene is mediated via co-suppression in which the introduced fragment leads to an overabundant production of sense mRNA which in turn leads to production of dsRNA via a pathway that is still not understood. The dsRNA produced by transcription of the *gm-fad2-1* partial sequence acts as a trigger for the post-transcriptional control mechanism that then effectively results in the degradation of RNA from both the *gm-fad2-1* partial sequence and the endogenous gene. This basic scenario would be similar to that postulated for co-suppression of the chalcone synthase gene in Petunia (Napoli *et al.*, 1990), the first example of co-suppression in a plant system. Down-regulation of *fad2-1* in other systems has also been achieved through other genetic modification mechanisms including nuclear retention of antisense transcripts (Buhr *et al.*, 2002) and use of co-suppression hairpin RNA constructs (Stoutjesdijk *et al.*, 2002).

3.3.2 gm-hra *gene*

The second genetic element introduced into soybean 305423 is a 1,971 bp complete gene sequence that encodes a modified version of the soybean endogenous acetolactate synthase (ALS) protein (Falco and Li, 2003) which confers a degree of tolerance to ALS-inhibiting herbicides. It is therefore known as *hra* (*herbicide – resistant als*). It was generated by site-specific mutagenesis of the endogenous soybean *als* gene that gives rise to a protein differing by two amino acids (see Section 4.2). Additionally, the coding region of the *gm-hra* gene differs from the endogenous *als* gene by having 5 artificial codons (ie 15 nucleotides) added at the 5' N-terminal end as a consequence of the construction of the plasmid (Falco and Li, 2003). These codons are adjacent to a chloroplast transit peptide protein sequence (see Section 4.2) and are presumably removed with it during targeting of the HRA protein to the chloroplast.

Expression of the introduced *gm-hra* gene is under the control of a 1.3 kb fragment obtained from the 5' promoter region of a soybean *S-adenosyl-L-methionine synthetase* (*sams*) gene (Falco and Li, 2003). This region consists of a strong constitutive promoter together with a

[4] The term 'silencing' is used to refer to suppression of expression of a gene and its effect may range from partial to total reduction in production of the encoded gene product.

translational leader region interrupted by a 591 nucleotide intron. The *sams* promoter, while constitutive, is particularly active in developing seeds, seedlings and callus tissue and is therefore useful in driving expression of marker genes during selection of genetically modified plants/tissue at an early stage of the transformation procedure (Falco and Li, 2003). At the 3' untranslated region of the introduced gene is an mRNA termination region derived from the soybean *als* gene.

Also included in the PP17752A fragment are 3 x Flippase (FLP) recognition target (FRT) sequences that flank the *gm-hra* gene. FLP recombinase is an enzyme native to the 2 µm plasmid of *Saccharomyces cerevisiae* (Cox, 1988) and is active at an FRT site. When two of these FRT sites are present, the FLP enzyme creates double-stranded breaks in the DNA strands, exchanges the ends of the first FRT with those of the second FRT, and then reattaches the exchanged strands. This process can lead to deletion of the DNA which lies between the two FRTs and then facilitate directional targeting of another desired gene into the corresponding recombination sites (Baszczynski *et al.*, 2008). The presence of these sites does not result in expression of any novel proteins or alter expression of the gene that they flank; their purpose is only to facilitate gene removal/insertion in any potential future genetic modification experiments that may involve soybean 305423.

The *gm-hra* gene in soybean 305423 is used as a marker gene; in order to select potential transformants at the early stage of the transformation procedure. Soybean cells were incubated in a medium containing chlorsulfuron (a sulfonylurea herbicide) and only those cells containing a functional *gm-hra* gene (and also, potentially, the *gm-fad2-1* fragment) were able to grow.

3.4 Characterisation of the genes in the plant

Evaluation of insert copy number, insert integrity and presence/absence of plasmid backbone sequences was done by Southern blot analysis and sequence analysis of plant material from the T4 and T5 generations (see Figure 4) of soybean 305423. In addition to a negative control derived from DNA of non-GM cultivar 'Jack', the Southern blot analyses also included positive controls derived from DNA of plasmids PHP19340 and PHP17752.

3.4.1 Transgene copy number and insertion integrity

Leaf tissue from individual seedlings of the T4 generation was used for these analyses. The probes used in the Southern blot analyses were homologous to the promoter region, coding sequence and terminator region of each of the two linear fragments PHP19340A and PHP17752A. As these probes are also homologous to endogenous soybean genomic sequences, the negative control DNA from 'Jack' helped to discriminate between the two hybridisation possibilities. Based on the Southern blot analysis, it was determined that:

- multiple copies, both intact and truncated, of PHP19340A have been inserted into the genome of 305423 soybean comprising, in total
  - eight copies of the *Kti3* promoter
  - seven copies of the *gm-fad2-1* fragment and
  - five copies of the *Kti3* terminator.

  One copy of the PHP19340A fragment is intact and contains a complete *Kti3* promoter, *gm-fad2-1* partial sequence, and *Kti3* terminator. Four copies of the PHP19340A fragment have some truncation but contain portions of all three cassette elements. Two copies of the PHP19340A fragment retain only the *Kti3* promoter with the *gm-fad2-1* partial sequence.

  One copy of the *Kti3* promoter is associated with a small (495 bp) non-functional fragment of plasmid backbone DNA (see Section 3.4.2).

- a single, intact PHP17752A fragment has been inserted into the genome of 305423 soybean.

3.4.2 Plasmid backbone DNA analysis

Leaf tissue from 7 individual seedlings from each of the T4 and T5 generations was used for this analysis which was done to determine whether any plasmid backbone had been included in the linear fragments introduced to soybean 305423, and, if so whether any functional elements (ie the hygromycin resistance gene (*hyg*) and the plasmid origin of replication (*ori*) – see Figures 1 and 2) were present. As the backbones of plasmids PHP19340 and PHP17752 are identical, the same set of probes was used to detect any backbone from the two linear fragments. The origin of replication is a 370 bp *Hae II* fragment (Tomizawa *et al.*, 1977). Results indicated that while a 495 bp plasmid backbone fragment was associated with a truncated copy of the *Kti3* promoter in one of the insertions (Insertion 3 – see Section 3.4.3), this fragment did not contain either *hyg* or *ori*.

3.4.3 Physical map of the inserted DNA

Total genomic DNA from 305423 soybean was digested with restriction enzymes and either cloned into cosmid vectors to construct cosmid libraries or probed with two fragments derived from the genetic elements of PHP19340A. The libraries were screened using a *Kti3* promoter fragment as probe and unique clones were identified and characterised by PCR amplification. Bands that hybridised with probes derived from PHP19340A were excised, cloned into a plasmid vector and characterised by plasmid libraries and inverse PCR. From these two approaches a total of four insertions (each with 5' and 3' genomic border sequences) were identified as follows:

- Insertion 1: one intact PHP19340A, one intact PHP17752A, and three truncated PHP19340A fragments (one with all three elements and two with only the *Kti3* promoter and the *gm-fad2-1* partial sequence)
- Insertion 2: one truncated PHP19340A fragment (with all three elements)
- Insertion 3: one truncated copy of the *Kti3* promoter with a non-functional 495 bp fragment of the plasmid backbone
- Insertion 4: two inverted truncated copies of the PHP19340A fragment (with all three elements) connected by the *Kti3* terminators.

Insertions 1 and 3 also contain short sequences of 'filler DNA'[5] at junctions.

These results are supported by the Southern blot analyses described in Section 3.4.1. The sequence of each insert was derived through a standard method.

The 5' and 3' genomic border regions of Insertions 1, 2 and 3 were verified to be of soybean origin by PCR amplification and sequencing of the border regions from both soybean 305423 and control soybean samples. Furthermore, nucleotide sequence similarity analysis was performed using BLASTN (Basic Local Alignment Search Tool Nucleotide) against the National Center for Biotechnology Information (NCBI - available online at http://www.ncbi.nlm.nih.gov/).GenBank nucleotide dataset. This showed significant identities of the 5' and 3; genomic border regions of the four insertions to public and proprietary soybean genomic sequences.

3.4.4 Open Reading Frame (ORF) analysis

The nature of the process by which genetic material is introduced into an organism may result in unintended effects that include the creation of new open reading frames (ORFs) in

[5] 'Filler DNA' is associated with non-homologous end joining in plants, a form of DNA strand break repair (Gorbunova and Levy, 1997).

the genome of the organism. These ORFs may, in turn, lead to the production of novel proteins which could have implications for human safety, particularly regarding toxicity and allergenicity, if ingested.

Using the sequence data encompassing the 5' and 3' genomic border sequences, each of the four insertions in soybean 305423 was screened for the presence of ORFs containing both start and stop codons that spanned any novel junctions and that were greater than or equal to 300 bp (100 amino acids) in length. Two such junction-spanning ORFs were identified as follows:

- #1 is located at the 5' insert/border junction of Insertion 2 and is 106 amino acids long with 9 amino acids contributed by the 305423 insertion.
- #2 extends out from the truncated *KTi3* promoter sequence into the 5' genomic border of Insertion 3, and is 235 amino acids long with 54 residues contributed by the 305423 insertion.

Results of toxin/allergen analysis of the proteins potentially coded by the ORFs are discussed in Section 4.1.

3.4.5 Conclusion about gene characterisation

Comprehensive Southern blot analysis and sequence data indicate that multiple intact and truncated copies of fragment PHP19340A have been inserted into 4 sites in soybean 305423 and that one intact PHP17752A fragment is co-located at one of these sites (Insertion1). Backbone analysis shows that while a length of plasmid backbone has been incorporated at one of the insertion sites it does not contain any functional elements. Two unexpected ORFs were found at junctions associated with two of the insertion sites.

3.5 Stability of the genetic changes

3.5.1 Generational stability

Generational stability was evaluated by studying the pattern of inheritance and segregation of the introduced genetic material in individual plants from the T4 (27 plants), T5 (30 plants) and segregating F2 (100 plants) generations. For this study, the F2 generation was derived from a cross with elite cultivar 'PHSB02'. DNA from cultivar 'Jack' was used as a control in all analyses, while DNA from cultivar 'PHSB02' was also used as a control for analysis of the F2 generation.

In Southern blot analysis of the genetic material using probes homologous to both the *gm-fad2-1* partial sequence and *gm-hra* gene (ie event-specific hybridisation), all insert-containing plants gave consistent results across the generations except in the case of a single recombinant in the 100 F2 individuals. The recombination event removed the PHP17752A fragment from Insertion 1. A further 1000 plants from three different segregating generations (BC1F2, BC2F2 and BC3F2) were analysed by PCR-based assay but failed to show any recombinants. This led to the conclusion that recombination had occurred at a very low frequency and that, essentially, the introduced DNA in soybean 305423 is stable across multiple generations.

3.5.2 Segregation analysis

Three different generations (F2, F3 and BC1F2) were analysed for Mendelian inheritance patterns of the PHP 19340A and PHP17752A introduced fragments. Because the fragments were co-transformed they were expected to have identical segregation ratios of the form 1:2:1 homozygous positive: hemizygous positive: homozygous negative (null). However, homozygous positive plants (+/+) were not differentiated from hemizygous positive plants

(+/-) and therefore the expected segregation ratio would be 3:1 positive (+/+ or +/-):negative (-/-). Three methods were used to score plants as either positive or negative:

- F2, F3 and BC1F2 generations – gas chromatography (GC) assay of seed. Those plants with seeds containing > 30% of total fatty acid as oleic acid were scored as positive.
- F2 generation – Southern analysis with *gm-fad2-1* probe. Extracts from those plants showing hybridisation with the probe were scored positive.
- BC1F2 generation – event and gene specific PCR assay. Event-specific PCR involved the use of a primer and probe set specific for soybean 305423, gene specific PCR involved the use of a primer and probe set specific to the *gm-hra* gene. Extracts from those plants in which probe hybridisation was observed were scored positive.

A chi-square statistical test was used to analyse whether there was any difference between the observed results and the results expected from Mendelian inheritance of the introduced genes. The results of the analysis (Table 2) were consistent with all four insertions being genetically linked and segregating at a single locus according to Mendelian principles.

Table 2: Comparison of observed and expected results (based on 3:1 ratio) from segregation of the introduced genes in soybean 305423.

Generation	Method	Observed		Expected		Chi-square test P-value[1]
		Positives +/+ or +/-	Negatives -/-	Positives +/+ or +/-	Negatives -/-	
F2						
Elite 1 background	GC[2]	73	27	75	25	0.729
Elite 1 background	GC followed by Southern analyses	76	24	75	25	0.908
F3						
Elite 1 background	GC	34	14	36	12	0.617
Elite 2 background		59	22	60.75	20.25	0.748
BC1F2						
Elite 1 background	Event and *gm-hra* gene specific PCR	111	33	108	36	0.630
Elite 2 background		74	24	73.5	24.5	1.000
Elite 1 background	GC	160	60	165	55	0.484
Elite 2 background		155	63	163.5	54.5	0.211

[1] P-values greater than 0.05 indicate no significant difference between observed and expected
[2] GC = gas chromatography

3.5.3 Conclusion about stability of the genetic change

Southern blot analysis of DNA from two selfed generations (T4 and T5) showed identical event-specific hybridisation patterns and confirmed that the introduced genes were stably incorporated into the genome of soybean 305423 in these generations. The same hybridisation pattern was observed in the F2 generation in all but one individual plant in which fragment PHP17752A had been removed by a recombination event. Since no other recombination events were identified in a further analysis of an additional 1000 segregating F2 plants, it was concluded that the introduced DNA is stable across multiple generations.

All four insertions identified in Section 3.4.3 are confirmed to be genetically linked and to segregate by Mendelian principles as a single locus.

3.6 The *fad2-1* partial sequence and gene silencing

The *gm-fad2-1* partial sequence is not expected to give rise to any protein product and the intent of its transcription in soybean 305423 is to decrease the expression of one of the endogenous soybean fatty acid desaturase genes (*gm-fad2-1*) that encodes microsomal ω-6 desaturase (see Section 3.3). No novel protein is therefore expected to be produced from transcript arising from the partial *gm-fad2-1* sequence.

To investigate the effectiveness of silencing of the endogenous *gm-fad2-1* gene in soybean 305423, Northern blot analysis was performed on soybean 305423 and 'Jack' mRNA isolated from developing seed (20 and 30 days after flowering) and leaf tissue of 10 plants, and probed with a sequence from the 3'UTR of the endogenous gene. Transcription analysis of two other endogenous soybean fatty acid desaturase genes *gm-fad2-2* and *gm-fad3*, was also performed. The *gm-fad2-2* gene is closely related to *gm-fad2-1* while the *gm-fad3* gene acts downstream of *gm-fad2-1* in the same fatty acid biosynthesis pathway (see Section 3.2). A summary of the probes used for Northern blot analysis is given in Table 3.

The insertion of a promoter fragment with homology to an endogenous gene can effectively silence expression of the endogenous gene (see eg Cigan *et al.*, 2005). *Kti3* transcription in seed was therefore also analysed by Northern blots to explore possible silencing of the endogenous *Kti3* gene due to the introduction of the *Kti3* promoter to soybean 305423 via the PHP19340A fragment.

The soybean endogenous *dapA* gene which encodes dihydrodipicolinate synthase, an enzyme involved in lysine biosynthesis and present in most tissues of the plant, was used as a control for RNA quality and method sensitivity.

Table 3: Probes used for expression analysis of endogenous genes in soybean 305423.

Probe name	Hybridisation target
***fad2-1* 3'UTR**	*fad2-1* transcript
***fad2-2* 3'UTR**	*fad2-2* transcript
fad3	*fad3* transcript
Kti3	*Kti3 transcript*
dapA	*dapA* transcript

The results of the Northern blot analyses showed the following:

- *gm-fad2-1* transcript was not detected in leaf tissue of either soybean 305423 or cultivar 'Jack'. This is expected as expression of the endogenous gene is restricted largely to seeds and expression of the introduced *gm-fad2-1* partial sequence is driven by a seed-specific promoter.
- *gm-fad2-1* transcript was present in seed from both soybean 305423 and cultivar 'Jack' but was significantly reduced in soybean 305423 seed, indicating that the endogenous *gm-fad2-1* gene has been silenced in soybean 305423.
- *gm-fad2-2* transcript was present at a low level in both soybean 305423 and cultivar 'Jack' seed. However, the level of *fad2-2* transcript in soybean 305423 seed was slightly lower than in cultivar 'Jack' seed indicating that the introduced *gm-fad2-1* fragment has had some suppression effect on the endogenous *gm-fad2-2* gene.

This is not unexpected because of the homology between the *gm-fad2-1* and *gm-fad2-2* genes.

- *gm-fad3* transcript was present at the same level in seed of both soybean 305423 and cultivar 'Jack', indicating that the introduced fragment had no suppression effect on the endogenous *gm-fad3* gene.
- *Kti3* transcript[6] was present in seed from both soybean 305423 and cultivar 'Jack' but was significantly reduced in soybean 305423 seed, indicating that the endogenous *Kti3* gene has been silenced in soybean 305423.

Conclusion

Northern blot analysis of RNA isolated from soybean 305423 indicated that endogenous *gm-fad2-1* and *Kti3* genes were both silenced in the seed as a result of insertion of multiple intact and truncated copies of fragment PHP19340A into 4 sites (as identified in Section 3.4) in soybean 305423.

3.7 Antibiotic resistance marker genes

No antibiotic marker genes are present in soybean 305423. Plasmid backbone analysis shows that no functional plasmid backbone has been integrated into the soybean genome during transformation ie the hygromycin resistance gene, which was used as a bacterial selectable marker gene, is not present in soybean 305423. The absence of the bacterial marker gene in the GM plant was confirmed by Southern hybridisation analysis as described in Section 3.3.

3.8 Conclusion about molecular characterisation

Soybean 305423 contains two introduced coding sequences and associated regulatory elements. The first is a 597 bp partial sequence of a soybean fatty acid desaturase gene (*gm-fad2-1*) and corresponds to approximately 40% of the middle region of the open reading frame of the endogenous gene. Molecular analysis indicates that, as intended, transcription of this partial sequence results in suppression of expression of the endogenous *gm-fad2-1* gene. The presence of the *Kti3* promoter that drives the *gm-fad2-1* partial sequence also causes some suppression of the endogenous *Kti3* gene; while not an intended outcome, this suppression is not unexpected.

The second coding sequence introduced into soybean 305423 is a 1971 bp complete gene sequence (*gm-hra*) that encodes a modified version of the soybean acetolactate synthase enzyme and confers a degree of tolerance to acetolactate-inhibiting herbicides. There are no antibiotic-resistance markers in soybean 305423.

Comprehensive molecular analyses of soybean 305423 indicate that there are 4 insertion sites at a single genetic locus. These sites contain multiple copies, both intact and truncated, of the *gm-fad2-1* partial sequence and a single copy of the *gm-hra* gene. Breeding over three generations has confirmed stability of the introduced genetic elements and segregation data indicate their Mendelian inheritance. Backbone analysis shows that while a length of plasmid backbone has been incorporated at one of the insertion sites it is not functional. Two unexpected ORFs are present at junctions associated with two of the insertion sites.

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[6] Since the *KTi3* coding region shares approximately 80% homology with two other endogenous genes, *Kti1* and *Kti2*, with similar transcript size it was not possible to distinguish which particular transcript the *Kti3* probe was hybridising with. However, since the *Kti3* gene is expressed predominantly in seeds while *Kti1* and *Kti2* are expressed mainly in leaves, roots and stems (Jofuku and Goldberg, 1989), it is highly likely that the Northern blot hybridisation using seed tissue reflects predominantly *K\ti3* transcription.

4. CHARACTERISATION OF NOVEL PROTEINS

In considering the safety of novel proteins it is important to consider that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects, although a small number have the potential to impair health, e.g., because they are allergens or anti-nutrients (Delaney *et al.*, 2008b). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, anti-nutritional and allergenic effects. To effectively identify any potential hazards requires knowledge of the characteristics, concentration and localisation of all novel proteins expressed in the organism as well as a detailed understanding of their biochemical function and phenotypic effects. It is also important to determine if the novel protein is expressed as expected, including whether any post-translational modifications have occurred.

Two types of novel proteins were considered:

- those that may be potentially generated as a result of the creation of ORFs during the introduction of the two linear DNA fragments (see Section 3.4.4)
- those that were expected to be directly produced as a result of the translation of the introduced genes. Since the *gm-fad2-1* partial sequence is not expected to give rise to any protein product, soybean 305423 produces only one novel protein (GM-HRA) from the introduced constructs. A number of different analyses were done to determine the identity, physiochemical properties, *in planta* expression, bioactivity and potential toxicity and allergenicity of the protein. Because the expression of proteins *in planta* is usually too low to allow purification of sufficient quantities for safety assessment studies, a heterologous bacterial expression system was used to generate large quantities of the GM-HRA protein. The equivalence of the bacterial-produced protein to the plant-produced protein was determined as part of the protein characterisation.

4.1 Potential toxicity/allergenicity of ORFs created by the transformation procedure

It is unlikely that either of the two ORFs identified in soybean 305423 (see Section 3.4.4) are able to undergo transcription and express a protein:

- #1 has a low degree of novelty (only 9 amino acids contributed by the insertion) and a low likelihood of transcription because of the absence of upstream and adjacent transcriptional elements.
- #2 has a low likelihood of transcription because the truncated *KTi3* promoter upstream is missing the elements necessary for transcription.

Even if transcription could occur, the protein products are unlikely to be of concern. The two ORFs were subjected to BLASTP (Basic Local Alignment Search Tool Protein) searches against the NCBI GenBank protein dataset, version 158, 15/2/07, in order to ascertain any similarities to potentially toxic or anti-nutritive proteins (for more detailed information of this type of analysis see Section 4.5.2). To ascertain if the ORFs had any potential for cross reactivity with known or putative allergens, they were screened against the FARRP7 allergen dataset (for more detailed information on this analysis see Section 4.6.2). No significant similarities of the two ORFs to any allergens, toxins or anti-nutrient proteins were found.

It is concluded that there are very low safety concerns relating to the two ORFs created by the transformation procedure used to generate soybean 305423.

4.2 Biochemical function and phenotypic effects of the GM-HRA protein

Non-GM soybean lines naturally contain a gene that encodes the enzyme acetolactate synthase (ALS). This enzyme is widely distributed in nature (Mazur *et al.*, 1987) and catalyses the first common step in the biosynthesis of the essential branched-chain amino acids isoleucine, leucine and valine (Figure 6). ALS-inhibiting herbicides such as sulfonylureas block this synthesis, causing rapid cessation of plant cell division and growth (Brown, 1990). However, changes (such as natural or induced mutation) to the amino acid sequence of ALS can result in tolerance to ALS-inhibiting herbicides (see Section 4.5.1).

Site-specific mutagenesis of the *als* gene resulted in the proline at position 183 of the amino acid sequence being changed to alanine and the tryptophan at position 560 being changed to leucine in the GM-HRA protein (Falco and Li, 2003). These changes confer on GM-HRA a degree of tolerance to ALS-inhibiting herbicides.

The full-length GM-HRA protein comprises 656 amino acids and has a predicted molecular weight of 71 kDa. However, as the coding region for the protein includes an N-terminal chloroplast peptide protein sequence that is cleaved from the protein during processing, the mature form of the protein contains 604 amino acids with a predicted molecular weight of 65 kDa.

Since GM-HRA activity, substituting for ALS activity in soybean 305423, may alter the availability of the substrates 2-ketobutyrate and pyruvate (Figure 6) other biochemical pathways that also require these substrates (eg fatty acid biosynthesis – see Figure 4) may also be affected (see Section 5.3.2).

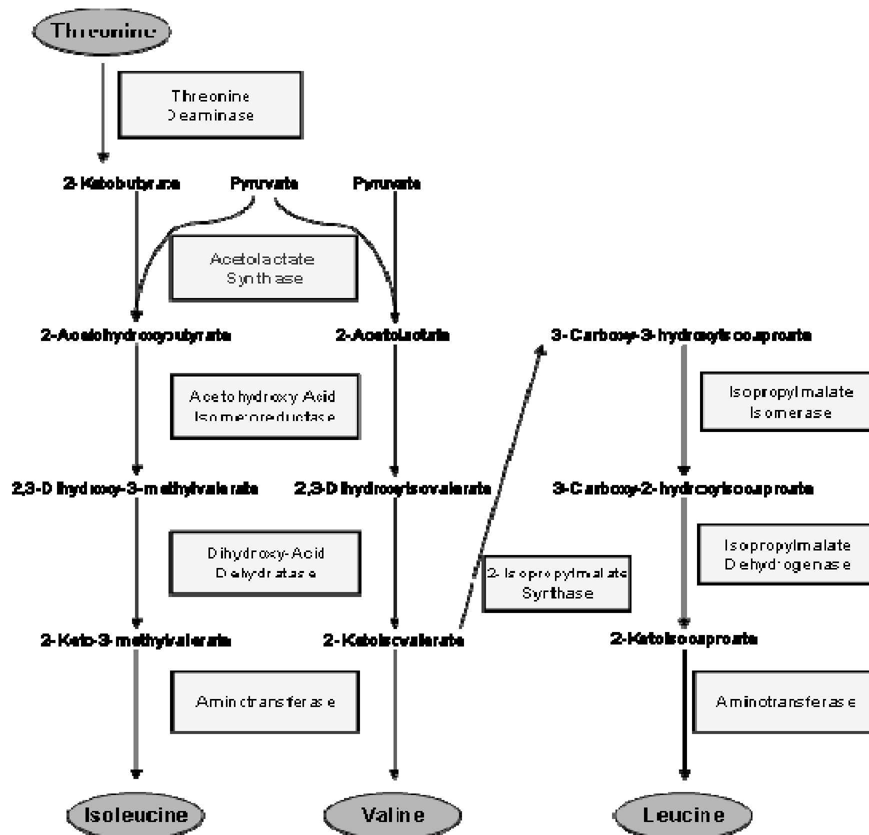

Figure 6. Branched chain amino acid synthesis in plants (adapted from Coruzzi & Last (2000)).

4.3 GM-HRA protein characterisation

A range of analytical techniques was used to determine the identity as well as the physicochemical and functional properties of the plant-derived GM-HRA protein isolated from soybean 305423 and to compare it to bacterially-derived protein.

Study submitted:

Buffington, J. (2006). Equivalency Assessment of the GM-HRA Protein derived from a Microbial Expression System with the GM-HRA Protein Derived from Soybeans Containing Event DP-305423-1. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-020 (unpublished).

Microbial GM-HRA protein was obtained as a fusion protein containing a His-T7 tag from a bacterial expression system (*Escherichia coli* BL21(DE3)RIPL). The His-T7 tag was cleaved from the affinity purified protein with thrombin and the purified protein was lyophilised. Characterisation of the purified protein was achieved through:

- the determination of identity using a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and scanning densitometry method, Western blot analysis, mass determination of tryptic peptides by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), N-terminal amino acid sequencing, amino acid composition analysis method and electrospray mass spectroscopy
- glycosylation analysis using a glycoprotein detection method.

Plant-derived GM-HRA protein was obtained from leaf tissue of soybean 305423 and partially purified by immunoaffinity chromatography using a mouse antibody that binds to both ALS and HRA proteins. Characterisation of the protein was done by:

- determination of identity using SDS-PAGE, Western blot analysis, mass determination of tryptic peptides by MALDI-MS and N-terminal amino acid sequencing
- glycosylation analysis using a glycoprotein detection method.

4.3.1 Protein identity

<u>Microbial- and soybean 305423- derived protein</u>

- SDS-PAGE analysis confirmed that the predominant protein band from both *E. coli* and soybean 305423 migrated at the expected 65 kDa.
- Western blot analysis, using ALS antibodies (recognising both endogenous ALS and HRA proteins) and GM-HRA-specific antibodies, detected an immunoreactive band migrating at approximately 65 kDa for both the bacterial- and soybean 305423-derived GM-HRA proteins.
- MALDI-MS analysis of the trypsin digest of the microbial GM-HRA protein identified 12 peptides that were within 100 ppm of theoretical peptide masses predicted from the *in silico* trypsin digest of the GM-HRA protein. In addition, 6 further peptide matches could be made allowing for minor modification of residues during processing for analysis. These 18 identified peptides account for 232/605 (38%) of the predicted amino acid sequence of GM-HRA. MALDI-MS analysis of soybean 305423-derived GM-HRA protein identified 10 peptides that were within 100 ppm of theoretical peptide masses predicted from the *in silico* trypsin digest of the GM-HRA protein. These peptides covered 118/604 (19.5%) of the predicted amino acid sequence of GM-HRA.
- N-terminal sequencing showed that the bacteria-derived GM-HRA protein contained an N-terminal glycine; this was expected as a consequence of thrombin cleavage of the His-T7 tag during protein purification. Otherwise, the next 12 amino acids were

consistent with the predicted sequence. The N-terminal sequence of the 12 amino acids of the mature form of the soybean 305423-derived GM-HRA protein was also as predicted.

Microbial-derived protein

- amino acid concentration analysis (using Beckman Model 7300 ion-exchange instrument) was done to determine the concentration of the bacteria-derived protein in the sample over 3 replicates. The actual concentration of HRA in the bacteria-derived extract was 29.1% (0.291 mg in 1 mg lyophilised powder).
- electrospray mass spectroscopy identified a major peak at 65,316 Da. This was consistent with the molecular mass of 65,312 Da for the mature HRA protein after accounting for the expected extra N-terminal glycine residue.

4.3.2 Glycosylation analysis

Glycosylation of a protein is a post-translational modification. Glycoproteins consist of carbohydrate moieties (glycans) covalently linked to a polypeptide backbone and the carbohydrate component may represent from <1% to >80% of the total weight. There is evidence that in transgenic plants, expression of non-native proteins may lead to inauthentic glycosylation and concomitant alteration of immunogenicity (see eg Prescott *et al*., 2005). Characterisation of protein glycosylation in genetically modified plants may therefore assist in informing the risk assessment process.

To assess whether post-translational glycosylation of the plant-derived GM-HRA protein occurred, glycosylation analysis of the purified protein sample from soybean 305423 was undertaken using a commercially available glycoprotein staining kit. Soybean trypsin inhibitor, a non-glycosylated protein was included as a negative control while horseradish peroxidase, known to be a glycosylated protein, was used as a positive control. The only protein to show glycoprotein staining was the horseradish peroxidase. No staining was detected for either the soybean trypsin inhibitor control or the plant-derived GM-HRA protein. The *E. coli*-derived GM-HRA protein was also confirmed to have no glycosylation. This was expected since *E. coli*, like most prokaryotes, lacks the capacity for protein glycosylation (Wacker *et al*., 2002).

4.3.3 Conclusion

A range of characterisation methods confirmed the identity and non-glycosylated status of GM-HRA protein produced in both a bacterial expression system and in soybean 305423. The protein from both sources was found to be equivalent and therefore it was concluded that microbially-derived GM-HRA protein is a suitable surrogate for use in safety assessment studies.

4.4 GM-HRA protein expression analysis

Study submitted:
Buffington, J. (2006). Protein Expression Analysis of Soybean Line DP-305423-1: U.S. and Canada Locations. Pioneer Hi-Bred International, Inc. Phase report ID: PHI-2005-002/010 (unpublished).

The GM-HRA protein levels were determined by enzyme linked immunosorbent assay (ELISA) using an HRA-specific antibody, in samples of leaf, root, forage (above ground parts that are harvested for use as feed for livestock), and seed tissues of soybean 305423, generation BC1F5, and control null segregant plants of generation BC1F5 (Figure 4). Leaf,

root and forage tissue was collected at the R3 growth stage[7] (when a pod is 5 mm long at one of the four uppermost nodes) while seed was collected at the R8 stage (95% of pods have reached mature pod colour). Tissues were collected from six separate field sites in North America[8] in 2005. Three replicated samples per tissue per site were collected for soybean 305423, and one sample per tissue per site was collected for control soybean. A commercially available software programme was used to calculate protein concentrations from optical density values.

The results (Table 4) indicate that no GM-HRA protein was detected in the non-GM controls but that all parts of soybean 305423 tested were found to express the GM-HRA protein, with forage containing the highest concentration. Seeds would be the part most likely to be used as food either directly or derived from seed by-products. The average content of GM-HRA protein in mature seeds of soybean 305423 is 2.5 ng/mg dry weight (range 0 – 4.9 ng/mg).

Table 4. Levels of GM-HRA protein in soybean 305423 (averaged across 6 sites).

Growth stage/ tissue	ng/mg tissue dry weight		Standard deviation
	Mean	Range[1; 2]	
	305423 soybean		
R3 / Leaf	4.0	1.2 – 6.3	1.8
R3 / Root	0.18	0 – 0.63	0.22
R3 / Forage	5.7	0.78 - 51	12
R8 / Seed	2.5	0 – 4.9	1.1
	Control soybean		
R3 / Leaf	0	0	0
R3 / Root	0	0	0
R3 / Forage	0	0	0
R8 / Seed	0	0	0

[1] Range denotes the lowest and highest individual values across sites.
[2] For values below the sample LLOQ (lower limit of quantitation), a value of zero was assigned for calculation purposes.

4.5 Potential toxicity of the GM-HRA protein

While the vast majority of proteins ingested as part of the diet are not typically associated with toxic effects, a small number may be harmful to health. Therefore, if a GM food differs from its conventional counterpart by the presence of one or more novel proteins, these proteins should be assessed for their potential toxicity. The main purpose of an assessment of potential toxicity is to establish, using a weight of evidence approach, that the novel protein will behave like any other dietary protein.

The assessment focuses on: whether the novel protein has a prior history of safe human consumption, or is sufficiently similar to proteins that have been safely consumed in food; amino acid sequence similarity with known protein toxins and anti-nutrients; structural properties of the novel protein including whether it is resistant to heat or processing and/or digestion. Appropriate oral toxicity studies in animals may also be considered, particularly where results from the biochemical, bioinformatic, digestibility or stability studies indicate a concern.

[7] For a full description of soybean growth stages see eg NSDU (2004).
[8] Sites were located in Richland (Iowa), Wyoming (Illinois), Paynesville, (Minnesota), York (Nebraska), Branchton (Ontario, Canada) and Thorndale (Ontario, Canada).

4.5.1 History of human consumption

The *gm-hra* gene was produced as a result of site-specific mutagenesis of a soybean *als* gene and encodes a protein differing from the native ALS protein by only two amino acids (see Section 4.1). The ALS enzyme is widely distributed (bacteria, fungi, algae and higher plants) in nature (Mazur *et al.*, 1987) and has therefore been inadvertently consumed in foodstuffs over a long period of human history.

Herbicides, such as the sulfonylureas, that target ALS in plants are commonly used worldwide and tolerance to ALS-inhibiting herbicides is common in weed species under selection pressure (see eg Bernasconi *et al.*, 1995). This tolerance is largely the result of point mutations within the *als* gene in which the substitution of a number of conserved amino acids in the encoded protein can confer resistance to ALS inhibitors (see reviews by Falco *et al.*, 1989; Tranel and Wright, 2002). Thus, while weed species may not be deliberately consumed, they have contributed to a level of background exposure to modified ALS proteins.

Commercially available soybean cultivars with tolerance to sulfonylurea have been developed where single amino acid changes have been introduced in the ALS enzyme using conventional means (DuPont, 2008; Sebastian *et al.*, 1989). Therefore, human consumption and exposure to modified ALS enzymes is not new.

4.5.2 Similarities with known protein toxins

Study submitted:

Cressman, R.F. (2006) Evaluation of the Amino Acid Sequence Similarity of the GM-HRA Protein to the NCBI Protein Sequence Datasets. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-071(unpublished).

Bioinformatic analyses are useful for assessing whether introduced proteins share any amino acid sequence similarity with known protein toxins. The GM-HRA (656 amino acids) sequence was compared with the non-redundant ("nr") protein sequence database available from NCBI (http://www.ncbi.nlm.nih.gov/). The Genpept "nr" dataset (Release 153.0 (4/15/06)) incorporates non-redundant entries from all Genbank nucleotide translations along with protein sequences from the SWISS-PROT, PIR, PRF and PDB databases. The NCBI database is a public database which, at the time the search was performed, contained over 3.6 million protein sequences, and thus provided a robust source from which to identify any potential protein toxin homologies.

The similarity search used the BLASTP (Basic Local Alignment Search Tool Protein) algorithm (Altschul *et al.*, 1997), now frequently applied for searching for similarities in protein sequences. The BLASTP algorithm searches for short stretches or domains of sequence similarity by performing local alignments. This detects more similarities that would be found using the entire query sequence length. A parameter known as the *E* value (see eg Baxevanis, 2005) represents the probability that a particular alignment is due to random chance. Comparisons between highly homologous proteins yield *E*-values approaching zero, indicating the very low probability that such matches would occur by chance. A larger *E*-value indicates a lower degree of similarity. All database sequences with an E-value of 1 or lower were identified by default by the BLASTP program. Although a statistically significant sequence similarity generally requires a match with an *E*-value of less than 0.01 (Pearson, 2000), setting a threshold *E*-value of 1.0 ensures that proteins with even limited similarity will not be excluded. Commonly, for protein-based searches, hits with *E*-values of 10^{-3} or less and sequence identity of 25% or more are considered significant although any

conclusions reached need to be tempered by an investigation of the biology behind the putative homology (Baxevanis, 2005).

The results from the search of the GM-HRA protein sequence returned 2000 entries with *E*-values below 1. Ninety five of the identified proteins returned E scores of zero and represent closely related ALS proteins from various plant species, including 31 ALS proteins from various crop plants. Other ALS proteins from various bacterial, archaebacterial and eukaryotic species account for another 922 of the protein matches. The remaining 1078 matches represent a variety of proteins that all possess one or more well-characterised, conserved thiamine pyrophosphate (vitamin B1) binding domains.

None of the proteins returned from the BLASTP search with the GM-HRA protein sequence are associated with known toxic or anti-nutritional properties therefore suggesting that the GM-HRA protein itself is unlikely to be a toxin or anti-nutrient.

4.5.3 In vitro digestibility

See Section 4.6.3

The digestibility of a protein has some relevance in a consideration of its toxic potential. since it provides evidence of any resistance to proteolytic enzymes. If a protein is digested quickly it is, in theory, less likely to be toxic or allergenic. However, *in vitro* digestion, by itself, does not provide an unequivocal conclusion on likely toxicity and other types of testing, particularly acute oral toxicity studies, should be used in conjunction. Digestibility has greater relevance to a consideration of allergenicity and the digestibility studies pertinent to soybean 305423 are therefore discussed in Section 4.6.3.

4.5.4 Thermolability

The thermolability of a protein provides an indication of the stability of the protein under cooking/processing conditions. It is a particularly relevant consideration in soybean-derived products since raw soybean cannot be consumed by humans because of the presence of anti-nutrient factors that are only destroyed by heat processing (OECD, 2001a).

Study submitted:

Comstock, B. (2007). Characterization of the Thermal Stability of GM-HRA Enzyme Activity. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-135 (unpublished).

GM-HRA protein obtained from a bacterial expression system (see Section 4.2) was incubated for 15 minutes at temperatures ranging from 36° – 60° C and tested for ALS activity using an indirect spectrophotometric assay that is associated with the enzyme product acetolactate. Following incubation of the enzyme with the substrate pyruvate, the end product acetolactate is converted to acetoin which is detected by the formation of a creatine and α-naphthol complex that is measured at 530 nm. To confirm the resistance of the GM-HRA protein to sulfonylurea herbicides, ALS activity was also measured in the presence of the herbicide chlorsulfuron.

The results (Figure 7) show a number of points:

- GM-HRA protein is able to use pyruvate as a substrate to produce acetolactate and therefore is similar to ALS in its function,
- unlike ALS, GM-HRA is resistant to a sulfonylurea herbicide – thus confirming the efficacy of the point mutations used to create the *gm-hra* gene
- GM-HRA is inactivated after incubation for 15 minutes at 50° C and is therefore unlikely to remain active following standard cooking/processing procedures.

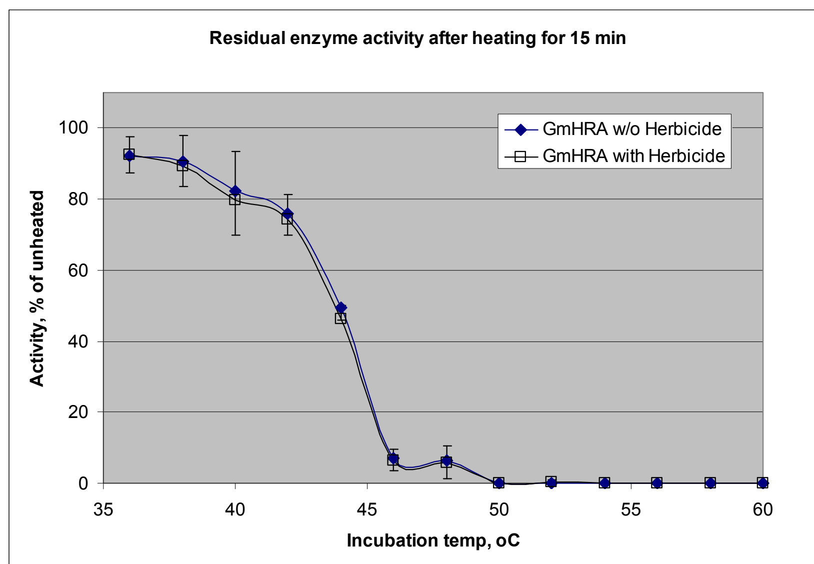

Figure 7. Response of HRA activity (in the presence and absence of a sulfonylurea herbicide) to heating.

4.5.5 Acute oral toxicity study

An acute oral toxicity study using mice was conducted to examine the potential toxicity of the GM-HRA protein obtained from a bacterial expression system (see Section 4.2).

Study submitted:

Finlay, C. (2006) GM-HRA: Acute Oral Toxicity Study in Mice. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-008 (unpublished).

Test material	GM-HRA preparation from *E. coli*
Vehicle	Deionised water
Test Species	Crl:CD®-1(ICR)BR mice (five males and five females, fasted)
Dose	2000 mg/kg body weight[9] HRA test substance by oral gavage (actual dose 436 - 582 mg/kg HRA since the purity of the protein is 29% in the test substance – see Section 4.3)
Control	Bovine serum albumin, 2000 mg/kg, or vehicle alone

Ten mice received a single dose of GM-HRA protein administered by oral gavage at an approximate dose of 582 mg/kg bw. Control groups of ten mice were administered bovine serum albumin at a dose of 2000 mg/kg, or water, once by oral gavage.

Mice were observed for mortality, body weight gain and clinical signs for 14 days. At the end of the study all animals were killed and examined post mortem for organ or tissue damage or dysfunction.

All mice survived through the duration of the study. No clinical signs of systemic toxicity were observed. No gross lesions were present in the mice at necropsy on day 14.

[9] The dose of 2,000 mg/kg body weight is the maximum unexceptional dose recommended by the OECD for the testing of acute oral toxicity using the fixed dose procedure (OECD, 2001b).

Under the conditions of this study, administration of GM-HRA to male and female mice at a dose of 582 mg /kg bw produced no test substance-related clinical signs of toxicity, body weight losses, gross lesions, or mortality. These results support the conclusion that the soybean HRA protein is not acutely toxic.

4.6 Potential allergenicity of the GM-HRA protein

The potential allergenicity of novel proteins is evaluated using an integrated, step-wise, case-by-case approach relying on various criteria used in combination, since no single criterion is sufficiently predictive of either allergenicity or non-allergenicity. The assessment focuses on: the source of the novel protein; any significant amino acid sequence similarity between the novel protein and known allergens; the structural properties of the novel protein, including susceptibility to digestion, heat stability and/or enzymatic treatment; and specific serum screening if the novel protein is derived from a source known to be allergenic or has amino acid sequence similarity with a known allergen. In some cases, such as where the novel protein has sequence similarity to a known allergen, additional *in vitro* and *in vivo* immunological testing may be warranted. Applying this approach systematically provides reasonable evidence about the potential of the novel protein to act as an allergen.

The allergenic potential of soybean HRA protein was assessed by:

- consideration of the *gm-hra* gene source and history of use or exposure
- bioinformatic comparison of the amino acid sequence of the GM-HRA protein with known protein allergen sequences
- evaluation of the lability of the microbially produced and purified GM-HRA protein from *E. coli* using *in vitro* gastric and intestinal digestion models; thermolability

4.6.1 Source of protein

The GM-HRA protein is derived from the native soybean ALS protein, differing only at two specific amino acids. While soybean is one of the eight major foods known to cause allergic effects (Metcalfe *et al*., 1996) and contains a number of proteins that have been identified as major allergens, ALS is not one of these proteins (Cordle, 2004; Csáky and Fekete, 2004; Ogawa *et al*., 2000). ALS proteins are present in many species, including other plant crop species and have not been associated with allergic reaction.

4.6.2 Similarity to known allergens

Study submitted:

Cressman, R. (2006) Comparison of the Amino Acid Sequence Identity between the GM-HRA Protein and Known Protein Allergens. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-070 (unpublished).

Bioinformatic analysis provides part of a 'weight of evidence' approach for assessing potential allergenicity of novel proteins introduced to GM plants. It is a method for comparing the amino acid sequence of the introduced protein with sequences of known allergens in order to indicate potential cross-reactivity between allergenic proteins and the introduced protein. As with the bioinformatic analysis that looked at similarities of GM-HRA with known protein toxins (see Section 4.4.2), the generation of an *E* value provides an important indicator of significance of matches (Baxevanis, 2005; Pearson, 2000).

To determine whether the GM-HRA protein has significant sequence identity to any proteins known or suspected to be allergens, the amino acid sequence of GM-HRA was compared to the Food Allergy Research and Resource Program (FARRP, University of Nebraska) Allergen Database (Version 6.0, January 2006) which contains the amino acid sequences of

known and putative allergenic proteins (www.allergenonline.com/about.asp) using established criteria (Codex, 2004). Potential similarities between the introduced protein in soybean 305423 and proteins in the allergen database were evaluated using the FASTA34 sequence alignment programme from the FASTA3 package (Pearson and Lipman, 1988). Alignments were inspected for identities greater than or equal to 35% over 80 or greater residues. The GM-HRA protein was also evaluated for any eight or greater contiguous identical amino acid matches to entries in the FARRP Allergen Database. These two approaches aim to identify both short contiguous regions of identity that could potentially correspond to shared IgE binding epitopes, as well as longer stretches of sequence similarity that may infer a potential cross-reactive protein structure.

Of 7 FASTA alignments between the GM-HRA protein sequence and the sequences in the FARRP Allergen Database, all had an *E*-value > 1. In addition, none of these exceeded the 35% threshold over 80 or greater amino acids. There were no eight or greater contiguous identical amino acid stretches in common between the GM-HRA protein sequence and any of the protein sequences in the allergen dataset. The results indicate that the GM-HRA protein does not show significant sequence identity with known allergens.

4.6.3 In vitro digestibility

Typically, food proteins that are allergenic tend to be stable to enzymes such as pepsin and the acidic conditions of the digestive system, exposing them to the intestinal mucosa and leading to an allergic response (Astwood and Fuchs, 1996; Kimber *et al*., 1999; Metcalfe *et al*., 1996). Therefore a correlation exists between resistance to digestion by pepsin and potential allergenicity. As a consequence, one of the criteria for assessing potential allergenicity is to examine the stability of novel proteins in conditions mimicking human digestion. Proteins that are rapidly degraded in such conditions are considered less likely to be involved in eliciting an response.

A pepsin digestibility assay was conducted to determine the digestive stability of the GM-HRA protein. In addition to the pepsin protocol using simulated gastric fluid (SGF), a second digestibility study was done using simulated intestinal fluid (SIF) containing pancreatin, which is a mixture of enzymes including amylase, trypsin, lipase, ribonuclease and protease. The relevance of the SIF study however is limited because ordinarily an ingested protein would first be exposed to pepsin-mediated hydrolysis in the acidic environment of the stomach before being subject to further digestion in the small intestine.

Simulated gastric fluid (SGF) study

Study submitted: Comstock, B. (2006) Characterization of the *In Vitro* Pepsin Resistance of GM-HRA. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-072 (unpublished).

The *in vitro* digestibility of the *E. coli*-derived GM-HRA protein in a prepared SGF (U.S.Pharmacopeia, 1995) containing pepsin at pH 1.2 was evaluated by SDS-PAGE (method based on Thomas *et al*., 2004). Digestibility of the protein in SGF was measured by incubating samples at 37° for selected times (0.5, 1, 2, 5, 10, 20, 30 and 60 minutes) and subjecting these to SDS-PAGE. Protein was visualised by Coomassie staining the resulting gel.

Two control proteins were treated in parallel: bovine serum albumin (BSA) is known to hydrolyse readily in pepsin and served as a positive control; β-lactoglobulin is known to persist in pepsin and was used as a negative control.

The GM-HRA protein was rapidly hydrolysed in SGF, with no GM-HRA protein detectable after 30 seconds exposure to SGF. The BSA positive control was also rapidly hydrolysed (< 1 minute) while the β-lactoglobulin negative control persisted for over 60 minutes.

Simulated intestinal fluid (SIF) study

Study submitted:

Comstock, B. (2006) Characterization of the *In Vitro* Pancreatin Resistance of GM-HRA. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-074 (unpublished).

The digestibility of *E. coli*-derived GM-HRA protein in SIF containing pancreatin (U.S.Pharmacopeia, 1995) was assessed using SDS-PAGE. Digestibility of the protein in SIF was measured by incubating samples with SIF containing porcine pancreatin, for specified time intervals (0, 0.5, 1, 2, 5, 10, 20, 30 and 60 minutes), and analysing by SDS-PAGE with protein staining, and also Western blot analysis.

Two control proteins were treated in parallel: bovine serum albumin (BSA) and β-lactoglobulin. The controls were incubated in SIF for 0, 1 and 60 minutes. Control proteins were detected by protein staining.

No visible HRA band was observed following Western blot analysis at one minute, indicating that the GM-HRA protein was rapidly (< 1 min) and completely hydrolysed in SIF containing pancreatin at pH 7.5.

The β-lactoglobulin positive control was also hydrolysed, with a faint band visible on a protein stained gel after one minute incubation, but no band visible after 60 minutes. The BSA negative control was not completely hydrolysed after 60 minutes.

4.6.4 Thermolability

See Section 4.5.4

4.7 Conclusion from characterisation of novel proteins

The introduced *gm-fad2-1* element is a partial sequence rather than a complete gene, therefore no novel protein is produced as a consequence of its transcription in cells of the GM soybean.

Soybean 305423 therefore expresses one novel protein – GM-HRA. The GM-HRA protein is 656 amino acids in length, has a predicted molecular weight of 71 kDa and differs from the endogenous soybean ALS by two amino acids at positions 183 and 560. The amino acid changes were generated by site-specific mutagenesis.

Expression analysis of the HRA protein has shown that it is expressed in leaves, roots, seeds and forage (above ground parts, including immature pods, that are harvested for use as feed for livestock). The average content in mature seeds is 2.5 ng/mg dry weight (range 0 – 4.9 ng/mg).

Extensive studies have been done to confirm the identity and physicochemical and functional properties of the expressed GM-HRA protein, as well as to determine its potential toxicity and allergenicity. The protein conforms in size and amino acid sequence to that expected and demonstrates the predicted enzymatic activity. It does not exhibit any post-translational modification including glycosylation and therefore is unlikely to be any different from ALS in its ability to elicit an immunogenic response.

Bioinformatic studies with the GM-HRA protein have confirmed the absence of any biologically significant amino acid sequence similarity to known protein toxins or allergens and digestibility studies have demonstrated that the protein would be rapidly degraded following ingestion, similar to other dietary proteins. Furthermore, the GM-HRA protein is heat labile. An acute oral toxicity study in mice with the GM-HRA protein has also confirmed the absence of toxicity and the near-identical ALS protein has inadvertently been consumed in food, including soybean, without apparent harm over a long period. Taken together, the evidence indicates that GM-HRA protein is neither toxic, nor likely to be allergenic, in humans.

5. COMPOSITIONAL ANALYSES

The main purpose of compositional analysis is to determine if any unexpected changes in composition have occurred to the food and to establish its nutritional adequacy. Compositional analysis can also be important for evaluating the intended effect where there has been a deliberate change to the composition of food.

The classic approach to the compositional analysis of GM food is a targeted one; rather than analysing every single constituent, which would be impractical. The aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health (eg solanine in potatoes)..

5.1 Key components

For soybean there are a number of components that are considered to be important for compositional analysis (EuropaBio, 2003; OECD, 2001a). As a minimum, the key nutrients of soybean seed appropriate for a comparative study include the proximates (crude protein, fat, ash, acid detergent fibre and neutral detergent fibre), amino acids and fatty acids. In addition, mineral and vitamin levels may be considered and international guidance also suggests that levels of the key anti-nutrients phytic acid, trypsin inhibitors, lectins, isoflavones and stachyose and raffinose should be determined for new varieties of soybean. The reasons for focussing on these particular anti-nutrients are:

- phytic acid causes chelation of mineral nutrients (including calcium, magnesium, potassium, iron and zinc) thereby making them unavailable to monogastric animals, including humans
- trypsin inhibitors interfere with digestion of protein; lectins are proteins that bind to carbohydrate-containing molecules. Both trypsin inhibitors and lectins can inhibit animal growth. The activity of trypsin inhibitors and lectins is heat-labile and they are inactivated during processing of soybean protein products and soybean meal so that the final edible soybean product should contain minimal levels of these anti-nutrients.
- isoflavones are reported to possess biochemical activity including estrogenic, anti-estrogenic and hypocholesterolaemic effects that have been implicated in adversely affecting animal reproduction. Major isoflavones in soybeans include daidzein, genistein, glycitein and coumestrol.
- stachyose and raffinose are low molecular weight carbohydrates (oligosaccharides) that are associated with production of intestinal gas and resulting flatulence when they are consumed.

5.2 Study design and conduct for key components

Studies submitted:

Maxwell, C. (2007). Nutrient Composition Analysis of Soybean Line DP-305423-1: U.S. and Canada Locations. Pioneer Hi-Bred International, Inc. Phase Report ID: PHI-2005-002/020 (unpublished).

Buffington, J. (2006). Agronomic Characteristics and Nutrient Composition Analysis of Commercial Non-Transgenic Soybean Lines: U.S. and Canada Locations. Pioneer Hi-Bred International, Inc. Study Id: PHI-2005-055/000 (unpublished).

Ideally, the comparator in compositional analyses should be the near isogenic parental line grown under identical conditions (OECD, 2001a). In the case of soybean 305423, the generation selected for testing was BC1F5 and the control was the BC1F5 null segregant (see Figure 4), and both were grown in 2005/2006 at six field locations in North America representative of the range of growing regions for soybean[10]. ELISA testing (sequential 'sandwich') of homogenised tissue from similar forage, leaf, root and seed batches of generation BC1F5 from soybean 305423 and the null segregant indicated that there was no detectable GM-HRA protein present in the null segregant samples (see Section 4.4) and therefore that inadvertent contamination of the control samples with GM material was unlikely to have occurred.

In addition, compositional analyses of four different non-GM commercial soybean cultivars, grown in six locations[11], provided additional comparators to establish reference ranges for compositional constituents. The reference cultivars were planted, harvested, processed and analysed using the same methods as used for soybean 305423 and the null segregant control line. Any statistically significant differences between soybean 305423 and the control could be compared to the reference range to assess whether the differences were likely to be biologically meaningful.

Plants were grown in a randomised complete block design comprising three blocks, each with two replicates at each location (ie 6 samples from each location). Seeds were collected at approximately the R8 growth stage (NDSU, 2004) with each sample containing approximately 300 g (fresh weight) of seed. Methods of composition analysis were based on internationally recognised procedures (e.g. those of the Association of Official Analytical Chemists), methods specified by the manufacturer of the equipment used for analysis, or other published methods.

Data from the commercial cultivars were used to calculate population tolerance intervals for each compositional component. Tolerance intervals are expected to contain, with 95% confidence, 99% of the values contained in the population of commercial lines. The population tolerance interval, together with the combined range of values for each analyte available from the published literature (ILSI, 2006; Kim *et al*., 2005; OECD, 2001a; Taylor *et al*., 1999), were used to interpret the compositional data for soybean 305423. Any mean value for a soybean 305423 analyte that fell within the tolerance interval and/or the combined literature range was considered to be within the normal variability of commercial soybean cultivars even if the mean value was statistically different from the null segregant control.

Statistical evaluation of the compositional data, using a standard computer programme, compared the seed from the soybean 305423 population to the null segregant control

[10] Sites were located in Richland (Iowa), Wyoming (Illinois), Paynesville, (Minnesota), York (Nebraska), Branchton (Ontario, Canada) and Thorndale (Ontario, Canada).

[11] Sites were located at Bagley (Iowa), York (Nebraska), Glen Allen (Virginia), Germansville (Pennsylvania), Larned (Kansas) and Branchton (Ontario, Canada)

population. Data were analysed using a linear mixed model design to account for the design effects of location and blocks within location. A least squares mean (LSM) value was generated and used for each analyte comparison.

In assessing the significance of any difference between the mean analyte value for soybean 305423 and the null segregant control, a P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

For those comparisons in which the soybean 305423 test result was statistically different from the control, the test mean was compared to the 99% tolerance interval derived from the commercial cultivars.

5.3 Analyses of key components

Although the Applicant provided results for the compositional analyses of forage, the focus of this assessment is necessarily on the food uses of soybean and therefore the forage data are not presented in this report. Compositional analyses of the soybean seed included proximates (protein, fat, and ash), acid detergent fibre (ADF), neutral detergent fibre (NDF), fatty acids, amino acids, isoflavones, anti-nutrients (stachyose, raffinose, lectins, phytic acid and trypsin inhibitor), minerals and vitamins.

5.3.1 Proximates and fibre

Results of the proximate and fibre analysis of soybean 305423 seed are shown in Table 5. Statistically significant differences between soybean 305423 and the null segregant control (P-value <0.05) were observed for fat and ash but the mean value for soybean 305423 for each of these analytes was within the range of values observed for the null segregant control as well as being within the statistical tolerance intervals for commercial soybean cultivars and the ranges reported in the literature (ILSI 2006; Taylor *et al.*, 1999). No statistically significant differences were observed between soybean 305423 and the control mean values for protein, ADF or NDF, and the mean values for these analytes were also within the statistical tolerance intervals and literature ranges.

Table 5. Percentage dry weight (dw) of proximates and fibre in soybean 305423 and control seed.

Analyte	Calculated variable	Control (% dw)	Soybean 305423 (%dw)	Tolerance interval	Combined literature range[1]
Protein	Mean (LSM)	40.7	41.2	29.9 - 48.7	32.0 - 47.4
	Range[2]	38.3 - 42.6	37.6 - 42.9		
	P-value[3]		0.1833[NS]		
Fat	Mean (LSM)	15.9	14.9	7.01 - 24.2	8.10 - 24.7
	Range	12.2 - 18.8	12.4 - 17.7		
	P-value		0.0377[***]		
ADF	Mean (LSM)	14.3	14.0	8.51 - 22.1	7.81 - 18.6
	Range	10.1 - 17.7	8.49 - 18.8		
	P-value		0.7108[NS]		
NDF	Mean (LSM)	13.5	13.6	8.07 - 21.9	4.50 - 21.3
	Range	9.91 - 16.9	9.61 - 17.7		
	P-value		0.7625[NS]		
Ash	Mean (LSM)	5.23	4.91	3.19 - 7.67	3.89 - 6.99
	Range	4.59 - 6.20	4.35 - 5.69		
	P-value		0.0001[***]		

[1] Literature ranges are taken from published literature for soybeans (ILSI 2006; Taylor *et al.*, 1999).
[2] Range denotes the lowest and highest individual value across locations.
[3] Probability values are significant at least at the 0.05% level ([*]) or are not significant ([NS])

5.3.2 Fatty acids

The levels of 25 fatty acids in soybean 305423 and null segregant control seed were measured, including the (9,15) isomer of linoleic acid as this isomer has been previously reported to occur in a GM high oleic soybean when the non-GM parent did not contain it (Kinney and Knowlton, 1997).

No data are presented for 11 fatty acids that were below the limit of quantitation. Results of the analysis for the 14 remaining fatty acids are given in Table 6 and confirm the expected high oleic acid and low linoleic and linolenic[12] acid levels in soybean 305423 as a result of the suppression of expression of the endogenous *gm-fad2-1* gene. The mean percentage composition for oleic acid (76.5%) was some 3.6x higher in soybean 305423 seed compared to the control (21.2%) while linoleic acid was 14.5x lower (3.62% in soybean 305423 compared with 52.5% in the control) and linolenic acid was 1.7x lower (5.39% in soybean 305423 compared to 9.35% in the control). The nutritional impact of these changes to fatty acid levels are discussed in Section 6. The level of linolenic acid in soybean 305423, while significantly lower than that in the control was, nonetheless within the normal range found in soybeans while the levels of oleic acid and linoleic acid in soybean 305423 were outside the normal range. The (9,15) isomer of linoleic acid was detected at similar percentage composition in both soybean 305423 and the control, although it was undetectable in the 4 reference cultivars. This isomer is present in a range of edible sources (including cheese, beef, hydrogenated vegetable oils and mango pulp) at concentrations ranging from 0.02% to 5.4% of total fatty acids (see discussion and references in Kinney and Knowlton, 1997).

In addition to these expected outcomes, there were differences between soybean 305423 and the null segregant control in terms of the following fatty acids:

[12] Lower linolenic acid levels are expected as a consequence of a reduction in the pool of linoleic acid that would be available for conversion to linolenic acid.

- the mean percentage composition of myristic acid, palmitic acid and stearic acid were all significantly ($P < 0.05$) lower in soybean 305423 compared to the control. However, all of the levels in soybean 305423 were within the statistical tolerance intervals.
- the mean percentage composition of palmitoleic acid, arachidic acid, eicosenoic acid and lignoceric acid were all significantly ($P < 0.05$) higher in soybean 305423 compared to the control. All of these levels except for eicosenoic acid were within the statistical tolerance range for the 4 commercial cultivars although the eicosenoic level was within the published range for this analyte.
- the mean percentage composition of both heptadecanoic acid and heptadecenoic acid was significantly ($P < 0.05$) higher in soybean 305423 compared to the control as well as being above the statistical tolerance range and the range reported in the literature. The Applicant has speculated that it is likely the increase in these two fatty acids is the result of expression of the GM-HRA protein causing a shift in availability of the GM-HRA enzyme substrates, pyruvate and 2-ketobutyrate (see Figures 5 & 6, and discussion in Section 4.2) which, in turn are substrates in the fatty acid biosynthetic pathway. The significance of the increased content of these two fatty acids is discussed below.

There was no difference between soybean 305423 and the null segregant control seed in terms of percentage composition of behenic acid.

Table 6. Percentage composition, relative to total fat, of major fatty acids in soybean 305423 and control seed.

Fatty acid	Calculated variable	Control (% total)	Soybean 305423 (% total)	Tolerance interval[1]	Range in lit[2]
Myristic acid (C14:0)	Mean (LSM)	0.0742	0.0451	0 - 0.174	0.0710 - 0.238
	Range[3]	0.0676 - 0.0807	0.0419 - 0.0522		
	P-value[4]		0.0001*		
Palmitic acid (C16:0)	Mean (LSM)	10.3	6.28	2.93 - 19.6	7.00 - 15.8
	Range	9.77 - 10.7	5.71 - 7.27		
	P-value		0.0001*		
Palmitoleic acid (C16:1)	Mean (LSM)	0.0860	0.0946	0.0110 - 0.177	0.0860 - 0.194
	Range	0.0751 - 0.0948	0.0835 - 0.105		
	P-value		0.0053*		
Heptadecanoic acid (C17:0)	Mean (LSM)	0.113	0.798	0.0722 - 0.131	0.0850 - 0.146
	Range	0.0993 - 0.127	0.703 - 0.890		
	P-value		0.0001*		
Heptadecenoic acid (C17:1)	Mean (LSM)	0.0614	1.19	0.0351 - 0.0732	0.0730 - 0.0870
	Range	0.0513 - 0.0762	1.01 - 1.51		
	P-value		0.0001*		
Stearic acid (C18:0)	Mean (LSM)	4.98	4.36	0.852 - 8.34	2.00 - 5.88
	Range	4.36 - 5.89	3.90 - 5.01		
	P-value		0.0001*		
Oleic acid (C18:1)	Mean (LSM)	21.1	76.5	11.3 - 32.6	14.3 – 34.0
	Range	18.0 - 24.1	68.7 - 79.4		
	P-value		0.0001*		
Linoleic acid (C18:2)	Mean (LSM)	52.5	3.62	41.7 - 64.3	42.3 - 60.0
	Range	50.2 - 54.3	1.53 - 8.98		
	P-value		0.0001*		

Fatty acid	Calculated variable	Control (% total)	Soybean 305423 (% total)	Tolerance interval[1]	Range in lit[2]
Linoleic acid (C18:2) isomer (9,15)	Mean (LSM)	0.247	0.341	Too low to measure	Not reported
	Range	0 - 0.532	0.143 - 0.456		
	P-value		0.0699[NS]		
Linolenic acid (C18:3)	Mean (LSM)	9.35	5.39	1.15 - 14.7	2.00 - 12.5
	Range	7.83 - 11.2	4.03 - 7.32		
	P-value		0.0001[*]		
Arachidic acid (C20:0)	Mean (LSM)	0.396	0.450	0.103 - 0.619	0 - 1.00
	Range	0.348 - 0.479	0.393 - 0.528		
	P-value		0.0001[*]		
Eicosenoic acid (C20:1)	Mean (LSM)	0.170	0.347	0.0549 - 0.319	0.140 - 0.350
	Range	0.135 - 0.201	0.290 - 0.394		
	P-value		0.0001[*]		
Behenic acid (C22:0)	Mean (LSM)	0.414	0.427	0.188 - 0.458	0.277-0.595
	Range	0.349 - 0.566	0.382 - 0.546		
	P-value		0.3779[NS]		
Lignoceric acid (C24:0)	Mean (LSM)	0.114	0.143	0 - 0.310	Not reported
	Range	0.0845 - 0.139	0.115 - 0.173		
	P-value		0.0003[*]		

[1] Negative tolerance limits were set to zero.
[2] Literature ranges are taken from published literature for soybeans (ILSI 2006; OECD, 2001a).
[3] Range denotes the lowest and highest individual value across locations.
[4] Probability values are significant at least at the 0.05% level ([*]) or are not significant ([NS])

Significance of compositional differences

a)

Both conventional breeding strategies and novel approaches (eg induced mutagenesis and genetic modification) have been successfully employed to significantly raise the oleic acid levels in a range of oilseed crops (see discussion and references in Liu *et al.*, 2002), some of which are now commercially available (Table 7). Of particular relevance is the approval of high oleic acid soybean for use in human food by Food Standards Australia New Zealand (FSANZ, 2000). The data in Table 7 show that the level of oleic acid in soybean 305423-derived oil is comparable to that in conventional olive, almond and avocado oils as well as being similar to, or lower than, the levels in oil derived from a number of specially bred high oleic acid commercial lines. The consumption of high levels of oleic acid is not considered to pose any safety concerns.

Table 7 also indicates that the level of linoleic acid in soybean 305423-derived oil is comparable to that in the oil derived from other specially bred commercial lines.

Table 7. Indicative oleic and linoleic acid content (% total oil) in a number of commercially available vegetable oils.

Source of oil	Oleic acid (% tot fat)	Linoleic acid (% tot fat)	Reference
High oleic soybean lines G94-1, G94-19 and G168	83.8	2.2	FSANZ (2000)
High oleic acid sunflower	83	7	Oilseeds International, Ltd (2002)
SunOleic® peanut	80	3	University of Florida (2003)
California high oleic safflower	77	15	Oilseeds International, Ltd (2002)
Soybean 305423	76.5	3.62	This application
High oleic canola	72.5	14	Dow AgroSciences (2009)
Olive (natural level)	72	9	Cordain (2002)
Almond (natural level)	69	17.4	Cordain (2002)
Avocado (natural level)	68	12.5	Cordain (2002)

b) Heptadecanoic (C17:0) and heptadecenoic acids (C17:1)

Together, these two fatty acids constitute approximately 2% of the total fatty acid content in soybean 305423, compared to approximately 0.17% in the control line 'Jack' (see Table 6). The potential for health risks caused by the increased levels of C:17 and C17:1 in soybean 305423 oil were considered by a) reviewing the metabolism of 17-carbon fatty acids in humans and animals, and b) assessing current exposure sources to the two fatty acids.

Metabolism of fatty acids

The degradation of fatty acids in humans and animals occurs in the mitochondria of cells in a cyclic process called β-oxidation. In this process, two carbon units are cleaved from the carboxy-terminus as acetyl-CoA subunits. Acetyl-CoA units are able to directly enter the tricarboxylic acid (TCA, or Krebs, cycle) to generate usable energy. In the case of fatty acids with an odd number of carbon atoms, such as C17:0 and C17:1, following the stepwise cleavage of acetyl-CoA units, the terminal metabolic substrate is a three carbon substance, propionyl-CoA. Propionyl-CoA is further metabolised to succinyl-CoA, a four carbon molecule, which also directly enters the TCA cycle.

Therefore, fatty acids such as C17:0 and C17:1 should be readily metabolised to TCA cycle intermediates and used in energy production.

C17:0 and C17:1 in the diet

The levels of C17:0 and C17:1 in soybean 305423 and the control line, 'Jack', were determined in refined, bleached and deodorised (RBD) soybean oil. These levels were compared to the levels reported in a range of foods reported in the USDA nutrition database (USDA, 2006). In addition, an analysis was done of fatty acids in oils and fats purchased from grocery stores in the U.S., using the same fatty acid detection methods used for RBD oil. Table 8 presents outcomes from this variety of sources. Levels of C17:0 and C17:1 in another GM soybean line (DP-356043-5) that has been approved for food use in Australia (FSANZ, 2009a) have also been included in Table 8 for comparison.

Table 8: Levels of C17:0 and C17:1 in soybean 305423 and some commonly consumed foods.

Food product	C17:0 (g per 100g)	C17:1 (g per 100g)
Soybean 305423 oil	0.8*	1.3*
Control Jack soybean oil	0.1*	0.03*
Soybean 356043 oil (FSANZ, 2009a)	0.26	0.15
Sunflower oil	0.04	Not reported
Corn oil	0.07*	Not reported
Olive oil	0.07*	0.13
Peanut oil	0.08*	Not reported
Unsalted sweet butter	0.66*	Not reported
Beef, ground (70% lean) raw	0.34	0.20
Tofu, extra firm	0.54	1.1
Lamb, cooked	0.30-1.2	Not reported
Cheese, pasteurised process	Not reported	0.2

[1] Serving sizes are 100 g for meat, tofu and cheese, and 30 g for oil and butter
* = level determined by detection of fatty acids in sample; other data are derived from USDA nutrition database except for Soybean 356043

These data demonstrate that C17:0 and C17:1 are typical constituents of the human diet. Heptadecanoic acid is present in vegetable oils, butter, tofu and meat, and heptadecenoic acid is present in beef, cheese, tofu and olive oil as well as in a commercially available GM-soybean oil. The estimated mean intake of C17:0 and C17:1 from soybeans for the US population represents approximately 0.2% of total fat intake per day (data derived from study reported in Section 6.1). The commercialisation of soybean 305423 may result in a minor increase in human dietary exposure to C17:0 and C17:1 fatty acids (see Section 6.1), however, no human safety or nutritional issues are expected as a result of this increase in exposure.

5.3.3 Amino acids

Levels of 18 amino acids were measured in seed from soybean 305423 and the null segregant control. Since asparagine and glutamine are converted to aspartate and glutamate respectively during the analysis, levels for aspartate include both aspartate and asparagine, while glutamate levels include both glutamate and glutamine.

As the GM- HRA enzyme is involved in branched chain amino acid biosynthesis of leucine, isoleucine and valine (see Section 4.2) it would not be unexpected that the levels of these amino acids, in particular, would differ between soybean 305423 and the control. Results (Table 9) show that levels of leucine, isoleucine and valine are not altered in soybean 305423 and that there is no difference between seed of soybean 305423 and the control in levels of any amino acid except threonine and glutamate (both of which are significantly higher in soybean 305423 although levels of both in soybean 305423 fall inside the tolerance interval).

Table 9. Percentage dry weight (dw), relative to total dry weight, of amino acids in soybean 305423 and control seed.

Amino acid	Calculated variable	Control (%dw)	Soybean 305423 (%dw)	Tolerance interval	Combined literature range[1]
Methionine	Mean (LSM)	0.714	0.712	0.488 – 0.852	0.431 - 0.681
	Range[2]	0.644 - 0.848	0.641 - 0.766		
	P-value[3]		0.9196[NS]		
Cystine	Mean (LSM)	0.638	0.614	0.378 – 0.869	0.370 - 0.808
	Range	0.489 - 0.730	0.554 - 0.689		
	P-value		0.1285[NS]		
Lysine	Mean (LSM)	2.56	2.58	1.98 – 3.10	2.29 - 2.86
	Range	2.34 - 2.76	2.27 - 2.83		
	P-value		0.7681[NS]		
Tryptophan	Mean (LSM)	0.496	0.507	0.359 – 0.632	0.356 - 0.670
	Range	0.449 - 0.597	0.436 - 0.605		
	P-value		0.3648[NS]		
Threonine	Mean (LSM)	1.91	1.95	1.57 – 2.21	1.14 - 1.89
	Range	1.78 - 2.02	1.77 - 2.06		
	P-value		0.0236*		
Isoleucine	Mean (LSM)	1.78	1.79	1.56 – 2.09	1.46 - 2.12
	Range	1.69 - 1.91	1.59 - 1.90		
	P-value		0.5729[NS]		
Histidine	Mean (LSM)	1.17	1.21	0.897 - 1.41	0.878 - 1.22
	Range	0.982 - 1.36	1.07 - 1.39		
	P-value		0.2893[NS]		
Valine	Mean (LSM)	1.84	1.87	1.58 - 2.18	1.50 - 2.44
	Range	1.72 - 2.01	1.66 - 2.02		
	P-value		0.36[NS]		
Leucine	Mean (LSM)	2.97	2.99	2.53 - 3.52	2.20 - 4.00
	Range	2.85 - 3.15	2.73 - 3.16		
	P-value		0.5439[NS]		
Arginine	Mean (LSM)	2.81	2.99	2.01 - 3.60	2.29 - 3.49
	Range	2.57 - 3.11	2.69 - 3.44		
	P-value		0.0723[NS]		
Phenylalanine	Mean (LSM)	2.07	2.10	1.74 - 2.43	1.60 - 2.35
	Range	1.92 - 2.28	1.87 - 2.23		
	P-value		0.5727[NS]		
Glycine	Mean (LSM)	1.89	1.93	1.54 - 2.18	1.46 - 2.02
	Range	1.75 - 2.05	1.77 - 2.06		
	P-value		0.3717[NS]		
Alanine	Mean (LSM)	1.66	1.73	1.35 - 2.07	1.49 - 2.10
	Range	1.50 - 1.82	1.47 - 1.98		
	P-value		0.2195[NS]		
Aspartate	Mean (LSM)	5.01	4.91	3.67 - 6.33	3.81 - 5.12
	Range	4.58 - 5.41	4.51 - 5.38		
	P-value		0.0653[NS]		
Glutamate	Mean (LSM)	7.69	7.92	6.04 - 9.54	5.84 - 8.72
	Range	6.87 - 8.48	7.49 - 8.38		
	P-value		0.0191*		

Amino acid	Calculated variable	Control (%dw)	Soybean 305423 (%dw)	Tolerance interval	Combined literature range[1]
Proline	Mean (LSM)	2.27	2.32	1.85 - 2.70	1.69 - 2.61
	Range	2.14 - 2.51	2.00 - 2.56		
	P-value		0.1805^{NS}		
Serine	Mean (LSM)	2.26	2.28	1.85 - 2.71	1.11 - 2.48
	Range	2.04 - 2.52	2.10 - 2.48		
	P-value		0.3492^{NS}		
Tyrosine	Mean (LSM)	1.34	1.36	0.908 - 1.69	1.02 - 1.62
	Range	1.13 - 1.59	1.14 - 1.48		
	P-value		0.6661^{NS}		

[1] Literature ranges are taken from published literature for soybeans (ILSI 2006; OECD, 2001a; Taylor *et al.*, 1999).
[2] Range denotes the lowest and highest individual value across locations.
[3] Probability values are significant at least at the 0.05% level (*) or are not significant (^{NS})

5.3.4 *Isoflavones*

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In total, there are 12 different soybean isoflavone isomers, namely the three aglycones genistein, daidzein , and glycitein, their respective β-glucosides genistin, daidzin, and glycitin, and three β-glucosides each esterified with either malonic or acetic acid (Messina, 2005). All 12 isomers were measured in seed from soybean 305423 and the null segregant control.

Levels of the 3 acetylglucosides were below the limit of quantitation and no data are presented. Levels for the remaining 9 isomers are given in Table 10 and show the following:

- the mean percentage dry weight of genistin, malonylgenistin, daidzin and malonyldaidzin in seed of soybean 305423 were significantly (P < 0.05) higher than in the control but were all within the tolerance intervals for the 4 commercial cultivars.
- there was no significant difference between seed of soybean 305423 and the control in terms of levels of genistin, genistein, daidzein, glycitin, glycitein and malonylglycitin.

Table 10. Weight (mg/kg total dry weight) of isoflavones in soybean 305423 and control seed.

Analyte	Calculated variable	Control (mg/kg dw)	Soybean 305423 (mg/kg dw)	Tolerance interval[1]	Combined literature range[2]
Genistin	Mean (LSM)	147	176	0 - 402	11.7 - 143
	Range[3]	88.9 - 225	106 - 308		
	P-value[4]		0.0362*		
Genistein	Mean (LSM)	11.1	12.2	0 - 32.3	0.5 – 22.6
	Range	<4.00[5] - 26.2	<4.00[5] - 37.1		
	P-value		0.4097^{NS}		
Malonylgenistin	Mean (LSM)	987	1100	0 - 2810	136 - 603
	Range	565 - 1540	740 - 1530		
	P-value		0.0003*		
Daidzin	Mean (LSM)	75.6	90.8	0 - 343	13.1 – 83.6
	Range	52.4 - 106	55.4 - 127		
	P-value		0.0055*		

Analyte	Calculated variable	Control (mg/kg dw)	Soybean 305423 (mg/kg dw)	Tolerance interval[1]	Combined literature range[2]
Daidzein	Mean (LSM)	13.0	12.6	0 - 47.1	0.1 – 21.2
	Range	<4.00[5] - 33.2	<4.00[5] - 49.7		
	P-value		0.9059[NS]		
Malonyldaidzin	Mean (LSM)	769	830	0 - 2880	61.9 - 558
	Range	535 - 1090	508 - 1110		
	P-value		0.007[*]		
Glycitin	Mean (LSM)	43.7	48.9	0 - 115	1.1 – 33.5
	Range	17.7 - 85.2	18.5 - 104		
	P-value		0.31[NS]		
Glycitein	Mean (LSM)	5.02	4.35	0 - 12.0	Not reported
	Range	<4.00[5] - 9.63	<4.00[5] - 5.60		
	P-value		0.4876[NS]		
Malonylglycitin	Mean (LSM)	114	119	0 - 295	6.6 – 71.2
	Range	57.7 - 206	55.4 - 238		
	P-value		0.3837[NS]		

[1] Negative tolerance limits were set to zero.
[2] Literature ranges are taken from published literature for soybeans (Kim *et al.*, 2005).
[3] Range denotes the lowest and highest individual value across locations.
[4] Probability values are significant at least at the 0.05% level ([*]) or are not significant ([NS])
[5] Values of the sample or samples were detected below the assay's Lower Limit of Quantitation (LLOQ). Sample results that were below the LLOQ are assigned a value equal to the LLOQ for statistical analysis.

5.3.5 Anti-nutrients

Levels of key antinutrients in seeds from soybean 305423 and the control are given in Table 11 and the following conclusions can be drawn:

- the level of stachyose was significantly (P < 0.05) higher in soybean 305423 than in the control but was within both the tolerance range for the 4 commercial soybeans and the range found in the literature.
- the level of trypsin inhibitor was significantly (P < 0.05) lower in soybean 305423 than in the control but was within both the tolerance range for the 4 commercial soybeans and the range found in the literature. The reduction in trypsin inhibitor may be explained by the suppression of expression of the endogenous *Kti3* gene as a consequence of the introduction of the *Kti3* promoter in soybean 305423 via the PHP19340A fragment (see Section 3.6). Equally, it may be due to normal variation.
- there was no significant difference between seed of soybean 305423 and the control in terms of levels of raffinose, lectins and phytic acid.

Table 11. Levels of anti-nutrients in soybean 305423 and control seed.

Analyte	Calculated variable	Control	Soybean 305423	Tolerance interval[1]	Combined literature range[2]
Stachyose (% dry weight)	Mean (LSM)	3.05	3.35	2.65 - 4.78	1.21 - 3.50
	Range[3]	2.57 - 3.52	2.46 - 3.81		
	P-value		0.0201[*]		
Raffinose (% dry weight)	Mean (LSM)	0.720	0.755	0 - 1.99	0.634 - 1.96
	Range	0.592 - 0.917	0.583 - 1.05		
	P-value		0.3619[NS]		

Analyte	Calculated variable	Control	Soybean 305423	Tolerance interval[1]	Combined literature range[2]
Lectins (hemagglutinating units/mg)	Mean (LSM)	3.06	3.65	0 - 11.4	0.105 - 9.04
	Range	0 - 8.53	1.24 - 7.48		
	P-value		0.0728[NS]		
Phytic acid (% dry weight)	Mean (LSM)	1.23	1.17	0.459 - 1.78	0.634 - 2.74
	Range	0.893 - 1.80	0.948 - 1.61		
	P-value		0.3637[NS]		
Trypsin inhibitor (trypsin inhibitor units/mg)	Mean (LSM)	50.2	32.9	8.71 - 80.4	19.6 - 119
	Range	43.1 - 59.9	28.2 - 36.9		
	P-value		0.0002*		

[1] Negative tolerance limits were set to zero.
[2] Literature ranges are taken from published literature for soybeans (ILSI 2006; OECD, 2001a)..
[3] Range denotes the lowest and highest individual value across locations.
[4] Probability values are significant at least at the 0.05% level (*) or are not significant (NS)

5.3.6 Minerals and vitamins

Mineral analysis was done for calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium and zinc (data not shown). Levels of calcium and magnesium were significantly ($P < 0.05$) lower in seed of soybean 305423 than in the control but the mean values were within the statistical tolerance intervals for the 4 commercial soybean varieties, and within the range of natural variation reported in the literature (ILSI 2006; OECD, 2001a).

Vitamin analysis was done for thiamin (B_1) riboflavin (B_2), folic acid, α-tocopherol, β-tocopherol, δ-tocopherol, γ-tocopherol and total tocopherols (data not shown). Levels of thiamin and γ-tocopherol were significantly ($P < 0.05$) lower in seed of soybean 305423 than in the control but the mean values were within the statistical tolerance intervals for the 4 commercial soybean varieties.

5.3.7 Conclusion of analysis of key components

Statistically significant differences in the analyte levels found in seed of soybean 305423 and the null segregant control are summarised in Table 12. It is noted that for eight analytes the soybean 305423 mean has fallen outside the reported range for the particular analyte but within the tolerance interval determined for four non-GM commercial cultivars. This suggests that the literature is somewhat limited in providing a broad reflection of the natural diversity that occurs within soybean.

The summary shows that, with the exception of oleic acid, linoleic acid, heptadecanoic acid and heptadecanoic acid the analyte levels found in soybean 305423 fall within the biological range found in non-GM commercial cultivars. The differences in levels of these four fatty acids are attributable to the two components of the genetic modification, ie the increase in oleic acid and decrease in linoleic acid are the result of suppression of the endogenous *gm-fad2-1* gene by the introduced *gm-fad2-1* fragment (see Sections 3.2 & 3.6), and the increase in heptadecanoic acid and heptadecenoic acid are likely to be the result of expression of the introduced *gm-hra* gene (see Section 4.2).

Table 12. Summary of analyte levels found in seed of soybean 305423 that are significantly (P < 0.05) different from those found in seed of the null segregant.

Analyte	Unit of measurement	Control mean	Soybean 305423 mean	Soybean 305423 within tolerance interval?	Soybean 305423 within reported range?
Fat	% dry weight	15.9	14.9	yes	yes
Ash	% dry weight	5.23	4.91	yes	yes
Myristic acid	% total fat	0.0742	0.0451	yes	no
Palmitic acid	% total fat	10.3	6.28	yes	no
Palmitoleic acid	% total fat	0.0860	0.0946	yes	yes
Heptadecanoic acid	% total fat	0.113	0.798	no	no
Heptadecenoic acid	% total fat	0.0614	1.19	no	no
Stearic acid	% total fat	4.98	4.36	yes	yes
Oleic acid	% total fat	21.1	76.5	no	no
Linoleic acid	% total fat	52.5	3.62	no	no
Linolenic acid	% total fat	9.35	5.39	yes	yes
Arachidic acid	% total fat	0.396	0.450	yes	yes
Eicosenoic acid	% total fat	0.170	0.347	no	yes
Lignoceric acid	% total fat	0.114	0.143	yes	Not reported
Threonine	% dry weight	1.91	1.95	yes	no
Glutamate	% dry weight	7.69	7.92	yes	yes
Genistin	mg/kg dry weight	147	176	yes	no
Malonylgenistin	mg/kg dry weight	987	1100	yes	no
Daidzin	mg/kg dry weight	75.6	90.8	yes	no
Malonyldaidzin	mg/kg dry weight	769	830	yes	no
Stachyose (% dry weight)	% dry weight	3.05	3.35	yes	yes
Trypsin inhibitor	units/mg	50.2	32.9	yes	yes
Calcium	% dry weight	0.241	0.190	yes	yes
Magnesium	% dry weight	0.248	0.223	yes	yes
Thiamin	mg/kg dry weight	1.86	0.981	yes	no
γ-tocopherol	mg/kg dry weight	112	88.7	yes	Not reported

5.4 Assessment of endogenous allergenic potential

Soybean naturally contains allergenic proteins and is one of a group of known allergenic foods including milk, eggs, fish, shellfish, wheat, peanuts, tree nuts and sesame. This group of foods accounts for approximately 90% of all food allergies (Metcalfe *et al.*, 1996). The presence of allergenic proteins in the diet of hypersensitive individuals can cause severe adverse reactions. The allergenic effect of soybeans is attributed to the globulin fraction of soybean proteins that comprise about 85% of total protein (OECD, 2001). Soybean-allergic individuals will also be allergic to soybean 305423.

Since soybean is associated with allergic effects in susceptible individuals, a study was done to assess whether seed from soybean 305423 may have an endogenous allergen content that was different from the non-GM parent line.

Study submitted:

Sampson, H. (2007). Evaluation of the IgE Binding of Conventional and 305423 Soybean Seeds using Sera from Soy Allergic Subjects. Pioneer Hi-Bred International, Inc. Study ID: PHI-2007-004, amended (unpublished).

Flour was prepared from seeds of soybean 305423 and the non-GM parent, 'Jack', and protein extracts were incubated with pooled sera from 5 soy-sensitive subjects (children and adults) for ID-IgE immunoblot and ELISA inhibition studies. These immunoassays are used routinely to identify protein-specific IgE binding by sera of individuals allergic to a particular food (Goodman and Leach, 2004; Ogawa *et al*., 2000). Negative controls were set up using commercially available normal (not atopic) human sera. In addition, single dimension SDS-PAGE Coomassie Blue stained protein profiles were obtained for the soybean 305423 and 'Jack' samples in order to compare their protein content.

The ID immunoblot data indicated that protein in soybean 305423 seed is similar in IgE binding profile to protein from 'Jack' seed. The ELISA inhibition data suggested close similarity in inhibition patterns between protein from soybean 305423 seed and that from 'Jack', in concentrations ranging from 50 to 500,000 ng/mL. The protein profiles of samples from the two sources were similar, indicating no difference in protein content between seed protein from soybean 305423 and 'Jack'.

Overall, these results suggest that protein from seed of soybean 305423 and 'Jack' is similar in both protein profile and allergen content. Thus, soybean 305423 appears to be equivalent to the non-transgenic counterpart in terms of its endogenous allergenicity.

5.5 Conclusion from compositional studies

Detailed compositional analyses were done to establish the nutritional adequacy of seed-derived products from soybean 305423 and to characterise the intended as well as any unintended compositional changes. Analyses were done of proximate (crude fat/protein, fibre, ash), amino acid, fatty acid, vitamin, mineral, sucrose, isoflavone, and anti-nutrient content. The levels were compared to levels in the non-GM parent and to those in four non-GM commercial soybean cultivars. These analyses indicated that the seeds of high oleic acid soybeans are significantly changed from those of the parental line with respect to their fatty acid profile.

The mean oleic acid content has been increased from 21.1% in the parental soybean to 76.5% in the high oleic soybean line, the linoleic acid and linolenic acid contents have been concomitantly decreased from a mean level of 52.5% to a mean level of 3.62% for linoleic acid and from 9.35% to 5.39% for linolenic acid. The level of linolenic acid in soybean 305423, while significantly lower than that in the control is, nonetheless within the normal range found in soybeans while the levels of oleic acid and linoleic acid in soybean 305423 are outside the normal range. The level of oleic acid in soybean 305423 oil is comparable to that in a range of other commercially available and commonly consumed vegetable oils. Consumption of such levels of oleic acid does not pose any safety concerns.

There has also been an unintended increase in two minor fatty acids, heptadecanoic acid and heptadecenoic acid. Together, these two fatty acids constitute approximately 2% of the total fatty acid content in soybean 305423, compared to approximately 0.17% in the control line. Both fatty acids are consumed as part of a normal human diet and are readily metabolised therefore the small increase in their levels is not considered to pose any safety concerns.

In terms of other analytes, seeds of soybean 305423 were found to be compositionally equivalent to those from the non-GM parent and other non-GM commercial soybean cultivars. Several minor differences in key nutrients and other constituents were found however, the mean levels observed are within the range of values observed for the non-transgenic comparator and within the range of natural variation.

In addition, no difference between soybean 305423 and the nontransgenic parent were found in allergenicity studies using sera from soybean-allergic individuals.

With the exception of oleic acid, linoleic acid, heptadecanoic acid and heptadecenoic acid, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key components in seed from soybean 305423 when compared with conventional soybean cultivars currently on the market.

6. NUTRITIONAL IMPACT

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and well being. In most cases, this can be achieved through an understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food.

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies using target livestock species will add little to the safety assessment and generally are not warranted (EFSA, 2008; OECD, 2003).

If the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the GM food, additional nutritional assessment should be undertaken to assess the consequences of the changes and determine whether nutrient intakes are likely to be altered by the introduction of such foods into the food supply. This assessment should include consideration of the bioavailability of the modified nutrient.

In the case of soybean 305423, there are significant changes in the levels of four fatty acids, (C17:0, C17:1, C18:1 and C18:2). These four fatty acids are typical constituents of the human diet.

The Applicant supplied data from a study in which the nutritional intake of a number of fatty acids was estimated for soybean oil derived from soybean 305423 and compared to known levels of consumption of soybean oil in the U.S. population[13]. As part of the assessment, FSANZ also undertook a similar dietary exposure assessment specifically for the Australian and New Zealand populations. The Applicant also submitted two animal feeding studies with soybean 3054323, the results of which are included below.

6.1 Quantitative dietary exposure assessment for soybean 305423 oil

The Applicant carried out the following *in silico* study (no formal study submitted).

Nutrient data for whole foods reported to be consumed in the U.S. were obtained from the National Health and Nutrition Examination Survey (NHANES), 1999-2002. This Survey does not provide data for heptadecanoic acid or heptadecenoic acid or the (9,15) isomer of linoleic acid and therefore calculations could be made only for palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and total *trans* fatty acids.

[13] Food consumption patterns in the U.S. are considered to be similar to those in Australia (WHO, 2009).

Dietary intake of total fat and fatty acids from soybean oil and dietary substitution data were calculated with FARE, a proprietary software program from Exponent, Food and Chemical Group (FARE version 7.99™, Durango Software, LLC, Bethesda, MD). Using FARE, the soybean oil portion of every food reported consumed in the NHANES database was assigned a nutritional profile. All data were statistically weighted according to the NHANES guidelines. Per capita nutrient intakes were calculated for the different population percentiles of the US population and four subpopulations (children 1-8, teens 9-19, adults 20-49, and adults 50+ years). Fatty acid intakes were evaluated using two scenarios: baseline (BL) and commercial adoption of oil from 305423 soybean (HO). Potential changes in the mean and 90th percentile of nutrient intakes from soybean oil alone were also estimated and allowed a comparison of BL and HO scenarios for the 3 fatty acids not included in NHANES (ie heptadecanoic acid, heptadecenoic acid, and the (9,15) isomer of linoleic acid).

For the total U.S. population, the substitution of soybean 305423 oil for existing soybean oil in a range of food applications resulted in a slight increase in intake of oleic acid and a slight decrease in both linoleic acid and *trans* fatty acids in the total diet (see Table 13). There was minimal effect on intakes of palmitic, stearic and linolenic acids. These trends were similar in all four subpopulations, although were slightly more pronounced in the teens 9 – 19 years category.

Table 13. Mean fatty acid intakes (g/person/day) estimated for non-GM soybean oil (BL) and oil from soybean 305423 (HO) in the total diet of the U.S. population.

Fatty acid	BL intake (mean g/day)	HO intake (mean g/day)	Net Effect of HO
palmitic	13.9	13.8	- 0.8%
stearic	7.0	7.0	0
oleic	23.4	25.0	+ 6.4%
linoleic	10.1	9.1	- 10%
linolenic	0.9	0.9	0
trans	5.9	5.5	- 6.8%

When the fatty acids were compared in terms of total soybean oil intake in the diet, the trends were similar to those shown in Table 11 but additionally there were slight increases calculated for heptadecanoic (0.005 – 0.04 g/day), heptadecenoic (0.001 – 0.06 g/day), and (9,15) isomer of linoleic acid (0.003 – 0.02 g/day) across the four subpopulations.

6.2 Dietary intake assessment for oleic and linoleic acids in the Australian and New Zealand populations

FSANZ undertook a baseline estimate for intake of each of oleic and linoleic acids in the Australian and New Zealand populations and compared this with a Scenario (Scenario 1) in which 25% of edible vegetable oils (excluding olive oil) were assumed to be soybean oil that was replaced with oil from soybean 305423, based on production data available for New Zealand (see Attachment 1). This is a conservative Scenario that is highly unlikely to occur for two reasons. Firstly, recent Australian oil industry data indicates soybean oil has an approximate market share of 2% of the Australian vegetable oil industry (excluding olive oil). Secondly, PLENISH™ high oleic acid soybean oil is not intended for retail sale and will be marketed to commercial producers that serve the food service and food processing industries ie it is not intended for applications at the residential or household level. Scenario 1 would be similar to the HO Scenario used in the U.S. study (Section 6.1). Concentrations of oleic and linoleic acid in foods used to calculate the baseline intake were derived from the food composition database NUTTAB (FSANZ, 2009b). Food consumption data

underpinning the assessment were taken from the 1995 Australian National Nutrition Survey (ABS, 1995) and the 1997 New Zealand National Nutrition Survey (Ministry of Health, 1999).

The results are summarised in Table 14 and indicate small changes in dietary intake of oleic (6% increase) and linoleic (10% decrease) acids between Baseline and Scenario 1. These are similar to those submitted by the Applicant for the U.S. population. Further details are given in Attachment 1.

Table 14. Estimated mean dietary intake of oleic and linoleic acids in Australian and New Zealand population groups under baseline and soybean 305423 scenarios.

Country	Population Group	Scenario	Mean Estimated Intake (g/day)	
			Oleic Acid	Linoleic Acid
Australia	2 – 6 years	Baseline	16.7	10.6
		Scenario 1	17.5	9.7
	2 years & above	Baseline	22.6	14.6
		Scenario 1	23.5	13.6
New Zealand	15 years & above	Baseline	25.3	16.4
		Scenario 1	26.9	14.7

6.3 Nutritional considerations of oleic and linoleic acids

In absolute terms, inclusion of oleic acid and linoleic acid (and other unsaturated fatty acids) in the diet is considered to offer health and nutritional advantages (see eg Liu *et al*., 2002; NH&MRC, 2006; Terés *et al*., 2008), particularly in relation to the lowering of LDL-cholesterol and concomitant reduction in the risk of cardiovascular disease, when compared to saturated fats (Mensink and Katan, 1992). It is significant to note that, while the levels of oleic and linoleic acids have been altered, the total percentage of unsaturated fatty acids is approximately the same in the control (83.26%) and in soybean 305423 (87.13%) (figures derived from data in Table 6).

There have been few studies that have specifically compared the effects of a change in diet from one high in linoleic acid to one high in oleic acid. Ryan et al. (2000) found that such a change in the diet of subjects with type 2 diabetes reduced insulin resistance and restored endothelium-dependent vasodilation. They suggested that this provided at least a partial explanation for the anti-atherogenic benefit of a Mediterranean-type diet where olive oil (with high oleic acid content – see Table 12) is the main source of fat (Visioli and Galli, 2001). In terms of both cooking quality and nutrition, the replacement of linoleic acid by oleic acid means that partial hydrogenation is not required to stabilise the fatty acids. This in turn, has the potential to reduce the intake of undesirable *trans* fats in the diet.

There is no established reference health standard for oleic acid. In Australia and New Zealand, for linoleic acid, the Adequate Intake (AI) varies from 5 g/day in children under 3 years to 13 g/day in adult men (NH&MRC, 2006). The Acceptable Macronutrient Distribution Range (AMDR) for linoleic acid is from 4 or 5% to 10%, expressed as a percentage of total energy intake for all age groups. On average 65% respondents had linoleic acid intakes above the relevant AI at Baseline, and a slightly lower proportion (63%) for Scenario 1. Approximately 50% respondents had linoleic acid intakes within the ADMR at Baseline, with a slightly lower proportion at Scenario 1 (see Attachment 1 for further details).

6.4 Feeding studies

Two feeding studies using food derived from soybean 305423 have been performed. A 42-day broiler study to make a nutritional assessment was submitted as part of the original Application and was subsequently published. Details of a rat feeding study were published in late 2008.

6.4.1 Broiler feeding study (McNaughton et al., 2008)

Study submitted:

Delaney, B.; Smith, B. (2007). Nutritional Equivalence Study of Transgenic Soybean Line DP-305423-1: Poultry Feeding Study. Pioneer Hi-Bred International, Inc. Study ID: PHI-2006-064/050 (unpublished).

This 42-day study compared growth performance and carcass yield of Ross x Cobb broiler chickens fed diets containing processed fractions (dehulled/defatted toasted meal, toasted ground hulls and de-gummed, alkaline-refined oil) from seeds of the B1F6 generation of soybean 305423 (see Figure 5) with those fed diets obtained from the same processed fractions from 4 non-GM soybean lines (null segregant BC1F6 plus three commercial cultivars).

Broilers were housed 10 broilers per pen (5 males and 5 females) with 12 pens (replicates) per treatment with 120 broilers in each of the 5 soybean line treatments.

Diets were formulated to meet nutrient requirements of a typical commercial broiler diet (National Research Council, 1994). Diets were fed in three phases according to standard commercial poultry farming practice, with soybean fractions incorporated at 26.5% meal for starter diets (days 0-21), 23% meal for grower (days 22-35) and 21.5% meal for finisher (days 36-42). Hulls and oil were added at 1.0% and 0.5% respectively to all diets. Feed and drinking water were available *ad libitum* throughout the study.

Birds were observed three times daily for overall health, behaviour and/or evidence of toxicity. Body weights and feed weights were determined every seven days. Body weight gain, feed intake and mortality-corrected feed:gain ratio (food efficiency[14]) were calculated daily for the duration of the study. At study termination, all surviving birds were processed to collect carcass and carcass part yield data. Carcass yield, thighs, breasts, wings, legs, abdominal fat, kidneys and whole liver were harvested for four males and four females per pen across each treatment (480 broilers).

Mean values from soybean 305423 and the null segregant control were generated for each trait to test the hypothesis that growth performance and carcass yield were different between broilers fed diets containing soybean 305423 and those fed diets derived from the non-GM control. A mixed model analysis of variance was used for statistical analysis and differences between means were considered significant at $P < 0.05$. Data generated from broilers fed fractions from the three non-GM commercial (reference) cultivars were used to construct a 95% tolerance interval containing 99% of observed values. If an observed value for a treatment group was contained within the tolerance interval, that value was considered to be similar to feeding 'typical' soybean diets.

No statistically significant differences were observed in growth performance (body weight gain, mortality, feed efficiency), organ yield (liver and kidney), or carcass yield (breast, thigh, leg (males), wing, and abdominal fat) variables between broilers consuming diets prepared

[14] Calculated as g of feed intake per g of body weight gain.

with isolated fractions from seeds of soybean 305423 or those from the null segregant control. However, within females, leg yield was significantly ($P < 0.05$) higher in the soybean 305423 test group (mean = 14.77%) than in the control group (mean = 14.26%). The mean value obtained for individuals in the soybean 305423 test group fell within the tolerance range (10.98% - 17.97%) calculated from the 3 commercial varieties. All other performance and carcass variables from control and soybean 305423 treatment groups also fell within tolerance intervals constructed for each response variable using data from broilers fed diets prepared with fractions from the three reference cultivars.

Based on the results from this study, it was concluded that seeds from soybean 305423 were nutritionally equivalent to those from the non-GM control soybean with a comparable genetic background.

6.4.2 Rat feeding study (Delaney et al., 2008a)

The same seed fractions from the same five soybean lines described in the previous study were incorporated into rodent diets nutritionally comparable to PMI® Nutrition International, LLC certified Rodent LabDiet® 5002 and fed *ad libitum* to young adult Crl:CD(SD) rats (12/sex/group) for at least 93 days. A range of parameters were recorded over the course, or at the completion, of the study including body weight, food efficiency, ophthalmology observations, neurobehavioral evaluation, clinical pathology, organ/body weight ratios, and anatomic pathology.

Response variable values from animals in the group fed fractions from the null segregant control were compared with those from animals in the group fed fractions from soybean 305423. Data from animals fed fractions from the three non-GM commercial soybean cultivars were used to generate tolerance intervals indicative of natural variation for each response variable.

Compared with rats fed the non-GM control diet, no biologically relevant differences were observed in rats fed the soybean 305423-derived diet with respect to body weight/gain, food consumption/efficiency, mortality, clinical signs of toxicity, or ophthalmological observations. No test diet-related effects were observed on neurobehavioral assessments, organ weights, or clinical or anatomic pathology. The study concluded that seeds from soybean 305423 are as safe and wholesome as those from non-GM soybeans.

6.5 Conclusion

Dietary exposure assessments of the fatty acids contained in soybean indicate that the substitution of soybean oil with oil from soybean 305423 would have minimal effect on the intake of dietary significant fatty acids. At most, if soybean oil was replaced with the oil derived from soybean 305423, there may be a marginal increase (up to 6%) in intake of oleic acid and a marginal decrease (up to10%) in linoleic acid intake. In terms of both cooking quality and nutrition, the replacement of linoleic acid by oleic acid means that partial hydrogenation is not required to stabilise the fatty acids. This in turn, has the potential to reduce the intake of undesirable *trans* fats in the diet. It is significant to note that, while the levels of oleic and linoleic acids have been altered, the total percentage of unsaturated fatty acids is approximately the same in the control and in soybean 305423. Taken overall, it is concluded that use of oil from soybean 305423 would have minimal nutritional impact. This conclusion is consistent with that reached by FSANZ for a previous high oleic acid soybean application (FSANZ, 2000).

Two animal feeding studies, in chickens and rats, indicate that the high oleic acid soybeans are nutritionally adequate and equivalent to non-GM soybeans in their ability to support typical growth and well-being.

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Attachment 1 - A1018 – Food Derived from High Oleic GM Soybean 305423: Dietary Intake Assessment Report

Summary

A dietary intake assessment was undertaken to estimate the current and potential dietary intakes of oleic acid (C18:1) and linoleic acid (C18:2), given that the concentration of these two substances is altered in soybean 305423 compared to control or conventional soybeans such that the oleic acid content is increased and linoleic acid content is decreased. Dietary intakes of oleic acid and linoleic acid were calculated for the Australian and New Zealand populations and for the population sub-group of Australian children aged 2-6 years.

The food consumption data used were from the 1995 Australian National Nutrition Survey (NNS) and the 1997 New Zealand NNS. The concentration data were derived from NUTTAB Food Composition Tables (Food Standards Australia New Zealand, 2007) and the Application.

Two scenarios were examined in the assessment of this Application:

> *'**Baseline**'* - estimation of current oleic and linoleic acid intakes from food.
>
> '***Scenario 1 - 25% market share***' - estimation of intakes of oleic and linoleic acids from food, assuming 25% of edible vegetable oils (excluding olive) is soybean oil that is replaced with oil from soybean 305423. The market share selected was based on available production figures for edible oils in the New Zealand market and represents the maximum amount likely to be contributed by soybean oil to total edible vegetable oils (excluding olive oil). The actual market share in Australia is less than 25% due to higher canola oil production compared to New Zealand.

The predicted dietary intakes for *Scenario 1* were as follows:

- Mean oleic acid intakes were predicted to increase
  - By 4% from 23 g/day for Australians 2 years and above
  - By 6% from 25 g/day for New Zealanders 15 years and above
  - By 5% from 17 g/day for Australians 2-6 years

- Mean linoleic acid intakes were expected to decrease
  - By 7% from 15 g/day for Australians 2 years and above
  - By 10% from 16 g/day for New Zealanders 15 years and above
  - By 9% from 11 g/day for Australians 2-6 years

The major contributing food groups (≥5%) to the dietary intake of oleic acid for all population groups and *Scenarios* were:

- Edible oils and oil emulsions
- Meat and meat products
- Dairy products
- Breads and bakery products
- Fruit and vegetables

The major contributing food groups (≥5%) to the dietary intake of linoleic acid for all population groups and *Scenarios* were:

- Edible oils and oil emulsions
- Breads and bakery products
- Meat and meat products
- Fruit and vegetables

The contribution to the dietary intakes of oleic acid or linoleic acid from soybean oil alone could not be assessed as few people reported consuming soybean oil specifically in the Australia and New Zealand NNSs from which these dietary intake data were derived.

There are no nutrient reference values (NRVS) for oleic acid. Linoleic acid dietary intakes were compared to the relevant NRVS; the Adequate Intake (AI) and the Accepted Macronutrient Distribution Range (AMDR) for population groups.

- *Baseline*
  - Approximately 65% of respondents from all population groups had intakes of linoleic acid at or above the AI
  - For all population groups assessed 50% of respondents had intakes of linoleic acid within the AMDR of 4-10% of their total daily energy (35% below AMDR and 15% above AMDR)

- *Scenario 1*
  - A slightly lower proportion of respondents from each population group had intakes of linoleic acid at or above the AI (approximately 63% below the AI)
  - Approximately 50% of respondents from each population group had intakes of linoleic acid within the AMDR specified for their age and gender

Based on the dietary intake assessment, a minor increase in mean intakes of oleic acid of up to 6% was predicted for all population groups assessed should oil from soybean 305423 be introduced into the Australian and New Zealand food supplies and replace soybean oil. Intakes of linoleic acid were predicted to decrease for all population groups by up to 10% with slightly higher proportions of population groups having linoleic acid intakes below the AI or outside the AMDR when compared with *Baseline* dietary intakes.

Glossary and Abbreviations

Defined below are a number of different terms used specifically in relation to dietary intake assessments conducted by FSANZ and how they are used in this report. They may differ to how they are used by other risk assessors and dietary intake assessments outside FSANZ.

Term	Definition
90th percentile	A level at which 10% of the population or 10% of data points are above. This may be used in relation to intake to the food chemical, or food consumption amounts, as derived from a distribution. Whether it is related to intake or consumption will be clearly explained in the text.
Adequate Intake (AI)	The average daily nutrient intake level based on observed or experimentally-determined approximations or estimations of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate (NHMRC, 2006).
Acceptable Macronutrient Distribution Range (AMDR)	The AMDR is an estimate of the range of intake for each macronutrient for individuals (expressed as per cent contribution to energy), which would allow for an adequate intake of all the other nutrients whilst maximising general health outcome (NHMRC, 2006).
Consumption Amount	Refers to the amount of food consumed.
Consumer	A respondent in the NNS who ingests (i.e. is exposed to) the food chemical being assessed via food eaten.
DIAMOND	Dietary Modelling of Nutritional Data computer program used by FSANZ for conducting dietary intake assessments
Intake	The amount of a nutrient that is ingested by a consumer.
FSANZ	Food Standards Australia New Zealand
Mean	Arithmetic mean (unless otherwise specified)
NNS	National Nutrition Survey
Respondent	Any person included in the NNS. There were 13,858 respondents to the Australian 1995 NNS aged 2 years and above, and 4,636 respondents to the New Zealand 1997 NNS aged 15 years and above. This term may also be used to refer to the number of respondents within a particular sub-population group.

Background

A dietary intake assessment was undertaken to estimate the current and potential dietary intakes of oleic acid and linoleic acid, given that the concentration of these two substances is altered in soybean 305423 compared to control or conventional soybeans. The Applicant provided data on the fatty acid profile of soy oil derived from soybean 305423 (see Table 1 for details).

The Application describes the intended modification of the fatty acid profile of soybean 305423 to include an increase in the concentration of oleic acid and a decrease in the concentration of linoleic acid. The magnitude of changes in concentrations of the other fatty acids found in soybean 305423 were not deemed sufficient to justify a dietary intake assessment and were not assessed in this Application.

Table 1: Concentrations of fatty acids in soybean oil from a control soybean and soybean 305423 as provided by the Applicant

Food Name	Concentration (g/kg)	
	Control	**Soybean 305423**
Palmitic acid (C16:0)	108	62
Heptadecanoic acid (C17:0)	1	8
Heptadecenoic acid (C17:1)	0.3	13
Stearic acid (C18:0)	47	46
Oleic acid (C18:1)	218	784
Linoleic acid (C18:2)	534	21
Linoleic acid (C18:2) isomer (9,15)	1	4
Linolenic acid (C18:3)	8	34
Total trans fatty acids	1	1

Dietary Modelling

Dietary modelling is a tool used to estimate dietary exposures to food chemicals or intakes of nutrients from the diet as part of the risk assessment process. To estimate dietary exposures to food chemicals, records of what foods people have eaten are required in addition to information on how much of the food chemical is in each food. The accuracy of these estimates depends on the quality of the data used in the dietary models. Sometimes not all of the data required are available or there is uncertainty about the accuracy so assumptions are made, either about the foods eaten or about chemical levels, based on previous knowledge and experience. The models are generally set up according to international conventions for food chemical intake estimates, however, each modelling process requires decisions to be made about how to set the model up and what assumptions to make; a different decision may result in a different answer. Therefore, FSANZ documents clearly all such decisions and model assumptions to enable the results to be understood in the context of the data available and so that risk managers can make informed decisions.

The dietary intake assessment for oleic and linoleic acids were conducted using dietary modelling techniques that combine food consumption data with nutrient concentration data to estimate intake of oleic acid and linoleic acid from the diet. The dietary intake assessment was conducted using FSANZ's dietary modelling computer program, DIAMOND.

Dietary intake = nutrient concentration x food consumption

Intake was estimated by combining usual patterns of food consumption, as derived from national nutrition survey (NNS) data, with current concentrations of oleic acid and linoleic acid in food, in addition to the concentrations in oil from soybean 305423.

Population Groups Assessed

The dietary intake assessment was conducted for both Australian and New Zealand populations. An assessment was conducted for the whole population (i.e. the whole nutrition survey population; Australians aged 2 years and above and New Zealanders aged 15 years and above), as well as for children aged 2-6 years (Australia only).

Oleic Acid and Linoleic Acid Concentration Levels

The concentrations of oleic and linoleic acid in foods used in the dietary intake assessment were derived from NUTTAB[15] (Food Standards Australia New Zealand, 2007). These foods and their corresponding oleic and linoleic acid concentrations are shown in Table A2.1 in Appendix 2. Data from the Applicant were used for scenario levels of oleic or linoleic acid for soybean oil (see Table 1). The concentrations used for soybean and other edible oils are shown in Table 2 and Table 3. Assumptions made are in the section *Assumptions in the Dietary Modelling*.

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[15] NUTTAB 2006 is FSANZ's most recent nutrient database. It contains data on the nutrient content of Australian foods. For further information see http://www.foodstandards.gov.au/monitoringandsurveillance/nuttab2006/index.cfm

Concentrations of oleic or linoleic acid were assigned to food groups using DIAMOND food classification codes. These codes are based on the Australia New Zealand Food Standards Code, namely Schedule 1 of Standard 1.3.1 – Food Additives. For example, the Schedule contains a section *8.3 Processed comminuted meat, poultry and game products* with an specific sub sections for 'fermented' versions of these products and for 'sausages and sausage meat'.

Scenarios for the Dietary Intake Assessment

Two scenarios were examined in the assessment of this application:

> ***'Baseline'*** - to estimate current oleic and linoleic acid intakes from food.
>
> '***Scenario 1 – 25% market share***' – to estimate dietary intakes of oleic and linoleic acids from food if 25% of all edible vegetable oils (except olive) were replaced with oil from soybean 305423.

Scenario 1 assumes that 25% of all edible vegetable oils (except olive) is soybean oil and that it is replaced with oil derived from soybean 305423. The market share selected was based on available production figures and import/export data for edible vegetable oils in the New Zealand market (Eyres, 2007) and represents the maximum amount likely to be contributed by soybean oil to total edible vegetable oils (excluding olive oil). The actual market share in Australia is less than 25% due to higher canola oil production compared to New Zealand[16]. The other edible vegetable oils included polyunsaturated, corn, grapeseed, safflower, sesame, sunflower, canola, almond, peanut and unspecified vegetable oils.

Olive oil was excluded from this group as it is assumed that it is deliberately selected for use in cooking or processing and would not be substituted with another oil. Although soybean oil is used in margarines, margarines were not included in the assessment as it was not known whether they consisted of non-soy blended oils (containing soy oil) or soy oil alone.

For all population groups assessed, the actual number of consumers of soybean oil was low in comparison to all respondents surveyed in the Australian 1995 NNS and the New Zealand 1997 NNS. Only 4 people (<1% of respondents) reported consuming soybean oil in the Australian population 2 years and above. For the New Zealand population 15 years and above, 42 people (9% of respondents) reported consumption. Australian children 2-6 years reported no consumption of soybean oil. For this reason, it was not possible to directly assess the impact of introducing oil from soybean 305423 on oleic and linoleic acid intakes by assuming only those people who consumed soybean oil consumers would consume oil from soybean 305423.

The estimate oleic and linoleic acid concentrations for all foods, except soybeans and edible oils, remained the same for *Baseline* and *Scenario 1* models (see Table A2.1 in Appendix 2).

See Figure 1 for more details on the dietary modelling approach.

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[16] Recent Australian oil industry data indicates soybean oil has an approximate market share of 2% of the Australian vegetable oil industry (excluding olive oil), therefore, a very conservative estimate of potential oleic and linoleic acid intakes were predicted for this assessment. In Australian products, soybean oil is also included in dressings, mayonnaise, margarine, frying oil, bottled oils, baby infant formula products, prepared meals and bakery product mixes.

Dietary Survey Data

DIAMOND contains dietary survey data for both Australia and New Zealand; the 1995 NNS from Australia that surveyed 13,858 people aged 2 years and above, and the 1997 New Zealand NNS that surveyed 4,636 people aged 15 years and above.

Both of the NNSs used a 24-hour food recall methodology. It is recognised that these survey data have several limitations (see the section *Limitations*).

Table 2: Mean concentrations of oleic and linoleic acid in oils used for the dietary intake assessment for Australia and New Zealand

Food Code	Food Name	Mean concentration (g/kg)			
		Oleic Acid		Linoleic Acid	
		Baseline	***Scenario 1****	***Baseline***	***Scenario 1****
2.1	Edible oils	347.4	456.6	461.4	351.3
2.1.3	Soybean oil	182.6	456.6	551.6	351.3

* Weighted mean concentrations, assuming 75% edible oils (excluding olive oil) and 25% soybean oil; soybean oil included in edible oil category for scenario 1 models.

Table 3: Concentrations of oleic and linoleic acid for all oils used in the dietary intake assessment

Food	Oleic Acid (%)	Linoleic Acid (%)
Polyunsaturated blended oil	29.1	47.5
Corn oil	27.2	54.1
Grapeseed oil	19.8	63.8
Safflower oil	13.4	72.3
Sesame oil	38.9	40.7
Sunflower oil	25.1	59.5
Canola oil	57.9	18.5
Almond oil	64.7	24.3
Peanut oil	42.5	33.3
Vegetable oil (not further specified)	29.1	47.5

Note: These concentrations were obtained from NUTTAB06 and then averaged to determine a concentration for 'Edible oils' shown in Table 2 above

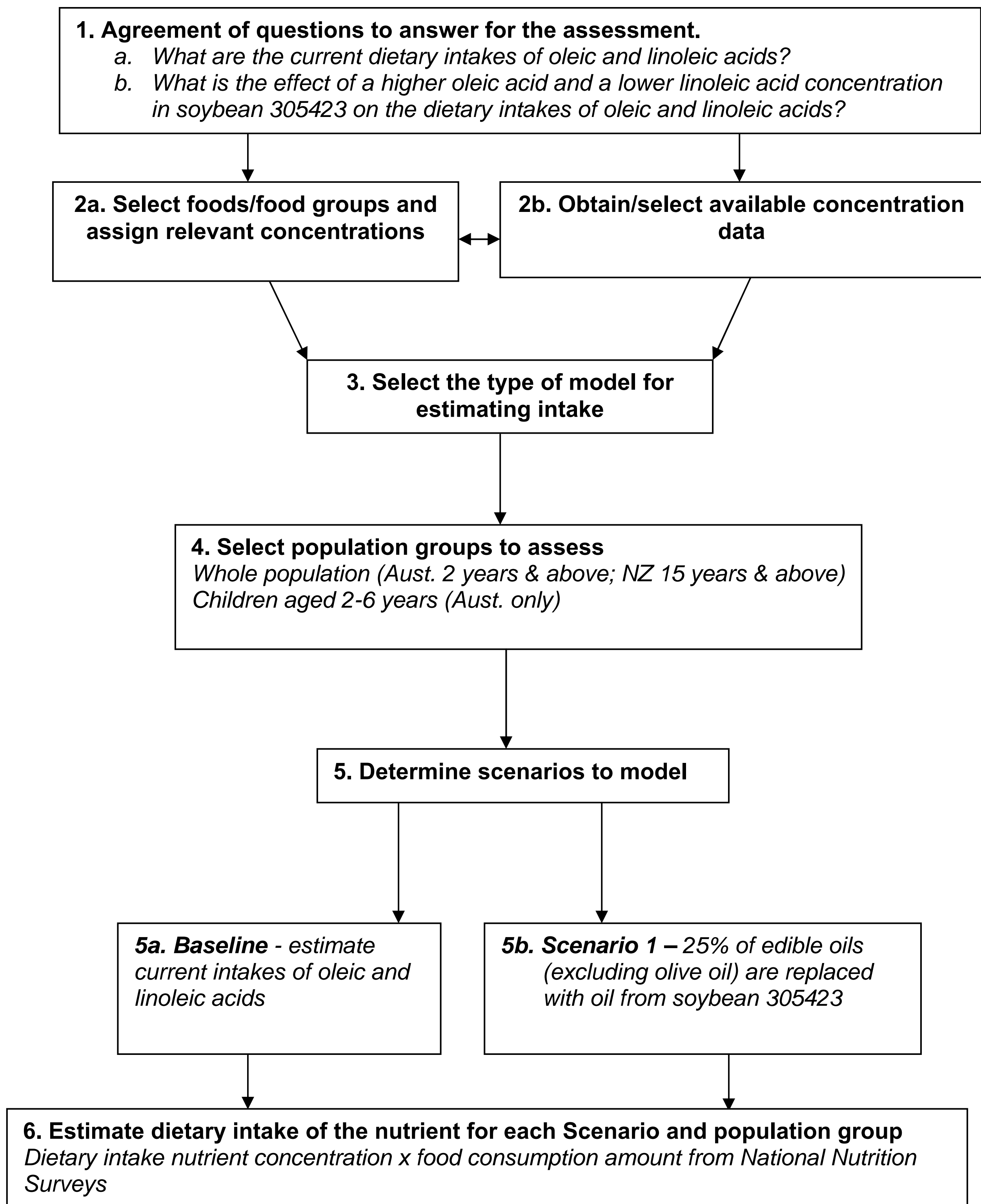

Figure 1: Dietary modelling approach used

How were the Estimated Dietary Intakes Calculated?

A detailed explanation of how the estimated dietary intakes were calculated can be found in Appendix 1.

Assumptions in the Dietary Modelling

The assumptions made in the dietary modelling are listed below.

Concentration data

- Soybean oil is assumed to have a 25% market share of edible vegetable oils (excluding olive oil) in Australia and New Zealand
- The oleic and linoleic acid content of margarines was assumed to be the same for *Baseline* and *Scenario 1*. Although soybean oil is known to be used in margarines, no data were available on levels of use of soybean oil in margarines available in Australia or New Zealand, so adjustments could not be made for the introduction of oil from soybean 305423 in margarines.
- All foods included in this dietary intake assessment contain oleic acid or linoleic acid at the levels specified in Table 2, Table 3 and Table A2.1 in Appendix 2.
- The oleic acid or linoleic acid concentrations in soybean 305423 in Australia and New Zealand food are the same as those found in foods overseas.
- Where a food was not included in the intake assessment, it was assumed to contain a zero concentration of oleic acid or linoleic acid.
- Where a food has a specified oleic or linoleic acid concentration, this concentration is carried over to mixed foods where the food has been used as an ingredient (e.g. oil in fried foods).

Consumption data

- Consumption of foods as recorded in the NNS represent current food consumption patterns.

Consumer behaviour

- Consumers do not alter their food consumption habits besides to substitute non-soybean 305423 containing products with soybean 305423 containing products.
- Consumers do not increase their consumption of foods/food groups upon foods/food groups containing soybean 305423 becoming available.

General

- Soybean 305423 is used only for oil and not any other soy product (e.g. soy protein isolate, dried soybeans, soy beverage, soy flour etc.) for the Australian and New Zealand food supplies.
- There is no contribution to oleic acid or linoleic acid intakes through the use of complementary medicines (Australia) or dietary supplements (New Zealand).

Results

Estimated Dietary Intakes of Oleic or Linoleic Acid

For all population groups examined only a small change in the estimated mean and 90th percentile dietary intakes for consumers of oleic acid or linoleic acid was predicted between *Baseline* and *Scenario 1.*

Oleic Acid

The estimated mean dietary intakes of oleic acid were predicted to increase from *Baseline* (22.6 g/day) to *Scenario 1* by 4% for Australians aged 2 years and above. Dietary intakes for New Zealanders aged 15 years and above were predicted to increase by 6% between *Baseline* (25.3 g/day) and *Scenario1*. For Australian children aged 2-6 years, a 5% increase in dietary intake of oleic acid was found between *Baseline* (16.7 g/day) and *Scenario 1*.

Dietary intakes for 90th percentile consumers predicted increases of between 6% (Australians 2 years and above) and 9% (Australians 2-6 years) for dietary intakes of oleic acid between *Baseline* and *Scenario1*.

See Figure 2 and Table A3.1 in Appendix 3 for all dietary intakes for the populations assessed.

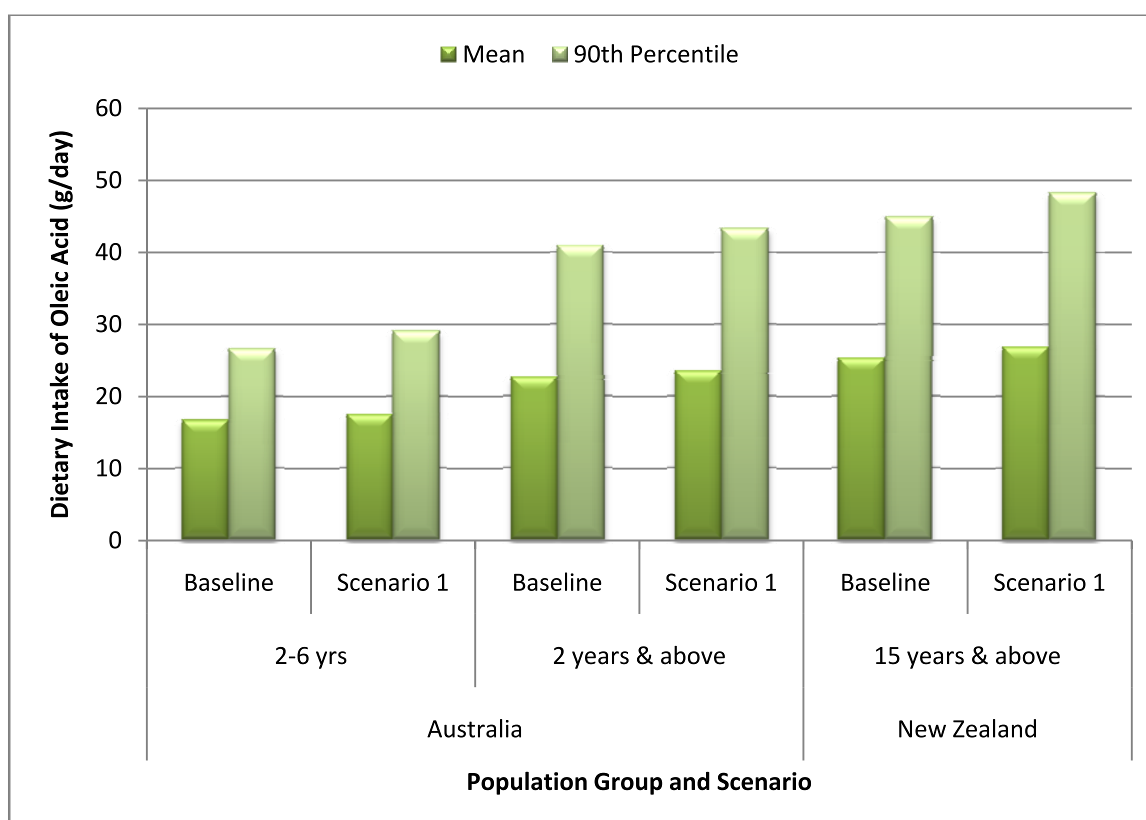

Figure 2: Estimated dietary intakes for consumers of oleic acid for Australian and New Zealand population groups

Linoleic Acid

The estimated mean dietary intakes of linoleic acid were predicted to decrease by 7% from *Baseline* (14.6 g/day) to *Scenario 1* for the Australian population 2 years and above.

A slightly larger 10% decrease in linoleic acid dietary intakes was predicted for the New Zealand population 15 years and above between *Baseline* (16.4 g/day) and *Scenario 1*.

For Australian children aged 2-6 years mean dietary intakes of linoleic acid decreased by 9% between *Baseline* (10.6 g/day) and *Scenario 1*.

Dietary intakes for 90th percentile consumers were predicted to decrease between 9% (Australians 2-6 years) and 12% (New Zealanders 15 years and above) for dietary intakes of linoleic acid between the *Baseline* and *Scenario 1*.

See Figure 3 and Table A3.2 in Appendix 3 for further details.

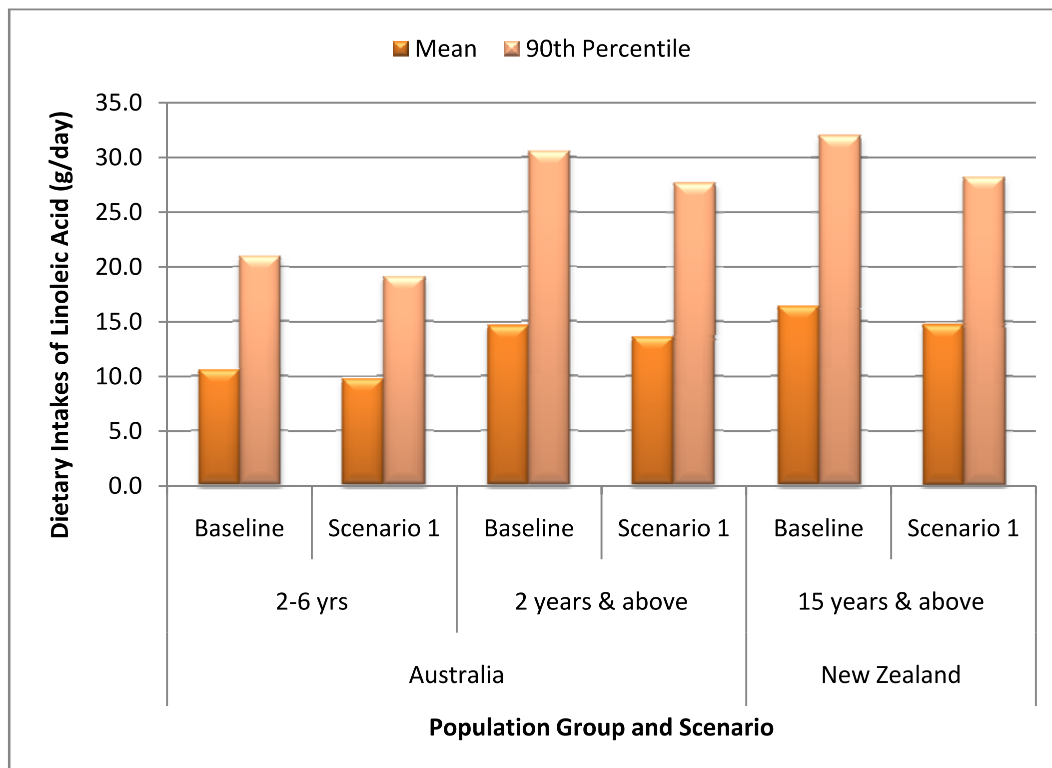

Figure 3: Estimated dietary intakes for consumers of linoleic acid for Australian and New Zealand population groups

Major Contributing Food Groups

Oleic Acid

For Australians aged 2 years and above, Australians 2-6 years and New Zealanders aged 15 years and above, edible oils and oil emulsions, meat and meat products, dairy products, breads and bakery products and fruit and vegetables were the major contributing food groups (≥ 5%) to oleic acid dietary intakes at *Baseline* and for *Scenario 1 (Tables 4, 5, 6)*. As expected soybean oil was not a major contributor to oleic acid intakes as there were very few respondents who reported consuming soybean oil specifically.

Further details on foods included in each food group can be found in Appendix 2. Food codes shown in the following tables correspond to food codes in Table A2.1.

Table 4: Major contributing foods to oleic acid dietary intakes for Australians aged 2 years & above

Major Food Group Code	Food Group	Percentage contribution to dietary intakes (%)	
		Baseline	***Scenario 1***
1	Dairy products (excluding butter and butter fats)	15	14
	Full fat milk	*6*	*6*
2	Edible oils and oil emulsions	34	37
	Oil emulsions (<80% oil)	*11*	*11*
	Margarine	*6*	*6*
	Edible oils	*14*	*17*
4	Fruits and vegetables	6	6
7	Breads and bakery products	13	13
	Plain breads	*5*	
8	Meat and meat products (including poultry and game)	23	22
	Poultry	*5*	*5*
	Beef and venison	*5*	*5*
	All other food groups	9	8

Note: The shaded cells indicate a contribution to dietary intakes of <5%, foods in *ITALICS* are included in the overall food group they belong to, however, an entry in this table indicates a contribution to the group total of ≥5% in their own right

Table 5: Major contributing foods to oleic acid dietary intakes for New Zealanders aged 15 years & above

Major Food Group Code	Food Group	Percentage contribution to dietary intakes (%)	
		Baseline	***Scenario 1***
1	Dairy products (excluding butter and butter fats)	13	12
	Full fat milk	*6*	*6*
2	Edible oils and oil emulsions	47	50
	Butter	*6*	*6*
	Margarine	*8*	*7*
	Oil emulsions (<80% oil)	*8*	*8*
	Edible oils	*21*	*25*
4	Fruits and vegetables	5	5
7	Breads and bakery products	15	14
	Plain breads	*5*	
8	Meat and meat products (including poultry and game)	12	11
	All other food groups	8	8

Note: The shaded cells indicate a contribution to dietary intakes of <5%, foods in *ITALICS* are included in the overall food group they belong to, however, an entry in this table indicates a contribution to the group total of ≥5% in their own right

Table 6: Major contributing foods to oleic acid dietary intakes for Australian children aged 2-6 years

Major Food Group Code	Food Group	Percentage contribution to dietary intakes (%)	
		Baseline	***Scenario 1***
1	Dairy products (excluding butter and butter fats)	23	22
	Full fat milk	*15*	*15*
2	Edible oils and oil emulsions	32	35
	Oil emulsions (<80% oil)	*9*	*9*
	Margarine	*6*	*6*
	Edible oils	*15*	*19*
4	Fruits and vegetables	5	5
7	Breads and bakery products	13	13
	Plain breads	*5*	
8	Meat and meat products (including poultry and game)	17	16
	Sausages	*5*	*5*
	All other food groups	10	9

Note: The shaded cells indicate a contribution to dietary intakes of <5%, foods in *ITALICS* are included in the overall food group they belong to, however, an entry in this table indicates a contribution to the group total of ≥5% in their own right

Linoleic Acid

For each population group assessed, the major contributing food groups (≥ 5%) to linoleic acid dietary intakes were similar for all scenarios. These food groups included edible oils and oil emulsions, breads and bakery products, and fruit and vegetables. For Australians 2 years and above and New Zealanders 15 years and above, meat and meat products were also a major contributing food group to dietary intakes of linoleic acid.

Further details can be found in Table 7, Table 8 and Table 9 below for major contributors and Table A2.1 in Appendix 2 for details on foods included in each food group.

Table 7: Major contributing foods to linoleic acid dietary intakes for Australians aged 2 years & above

Major Food Group Code	Food Group	Percentage contribution to dietary intakes (%)	
		Baseline	***Scenario 1***
2	Edible oils and oil emulsions	56	52
	Margarine	*25*	*27*
	Edible oils	*28*	*22*
4	Fruit and vegetables	16	17
	Nuts and seeds		*5*
	Fruit and vegetable preparations (eg. paste)	*9*	*10*
7	Breads and bakery products	13	14
	Plain breads	*10*	*10*
8	Meat and meat products (including poultry and game)	6	7
	All other food groups	9	10

Note: The shaded cells indicate a contribution to dietary intakes of <5%, foods in *ITALICS* are included in the overall food group they belong to, however, an entry in this table indicates a contribution to the group total of >5% in their own right

Table 8: Major contributing foods to linoleic acid dietary intakes for New Zealanders aged 15 years & above

Major Food Group Code	Food Group	Percentage contribution to dietary intakes (%)	
		Baseline	***Scenario 1***
2	Edible oils and oil emulsions	64	60
	Margarine	*17*	*19*
	Edible oils	*42*	*36*
4	Fruit and vegetables	11	12
	Fruit and vegetable preparations (eg. pastes)	*6*	*6*
7	Breads and bakery products	14	16
	Plain breads	*10*	*11*
	All other food groups	11	12

Note: The shaded cells indicate a contribution to dietary intakes of <5%, foods in *ITALICS* are included in the overall food group they belong to, however, an entry in this table indicates a contribution to the group total of >5% in their own right

Table 9: Major contributing foods to linoleic acid dietary intakes for Australian children aged 2-6 years

Major Food Group Code	Food Group	Percentage contribution to dietary intakes (%)	
		Baseline	***Scenario 1***
2	Edible oils and oil emulsions	57	54
	Margarine	*22*	*24*
	Edible oils	*32*	*27*
4	Fruit and vegetable preparations	16	17
	Fruit and vegetable preparations (eg. paste)	*10*	*10*
7	Breads and bakery products	13	14
	Plain breads	*10*	*10*
8	Meat and meat products (including poultry and game)	5	5
	All other food groups	9	10

Note: The shaded cells indicate a contribution to dietary intakes of <5%, foods in *ITALICS* are included in the overall food group they belong to, however, an entry in this table indicates a contribution to the group total of >5% in their own right

Limitations of the Dietary Modelling

Dietary modelling based on 1995 or 1997 NNS food consumption data provides the best estimate of actual consumption of a food for individuals and the resulting estimated dietary intake of a nutrient for the population. However, it should be noted that the NNS data do have limitations. These limitations relate to the age of the data and the changes in eating patterns that may have occurred since the data were collected. Generally, consumption of staple foods such as fruit, vegetables, meat, dairy products and cereal products, which make up the majority of most people's diet, is unlikely to have changed markedly since 1995/1997 (Cook *et al.*, 2001a; Cook *et al.*, 2001b). It is assumed that the NNSs give an underestimate of the realistic consumption of soybean oil as foods containing soybean oil as part of an oil blend or where blended oils are used in processing or for margarines were not specifically recorded in the NNS and are therefore not picked up through the dietary modelling for *Scenario 1*. The consumption of oil between 1995/1997 and today is not expected to have changed in terms of quantity, however, the type of oil consumed may have changed. It is likely that oils marketed as 'healthier' and higher quality will have increased in consumption, for example olive oil, in replacement of other types of oil. This implies that the fatty acid intakes estimated for this assessment could be different, for example, if consumers have switched from polyunsaturated oil to olive oil.

A limitation of estimating dietary intake over a period of time associated with the dietary modelling is that only 24-hour dietary survey data were available, and these tend to over-estimate habitual food consumption amounts for high consumers. Therefore, predicted high percentile intakes are likely to be higher than actual high percentile intakes over a lifetime.

A limitation of estimating dietary intake over a period time using information from a recall method is that people may over- or under-report food consumption, particularly for certain types of foods. Over- and under-reporting of food consumption has not been accounted for in this dietary intake assessment.

While the results of NNSs can be used to describe the usual intakes of groups of people, they cannot be used to describe the usual intake of an individual (Rutishauser, 2000). In particular, they cannot be used to predict how consumers will change their eating patterns as a result of an external influence such as the availability of a new type of food.

Statistical population weights were not applied to each individual in the NNSs. These weights make the data representative of the actual population as a whole. Maori and Pacific peoples were over-sampled in the 1997 New Zealand NNS so that statistically valid assessments could be made for these population groups. As a result, there may be bias towards these population groups in the dietary intake assessment because population weights were not used.

Risk Characterisation

Linoleic Acid

Two reference health standards have been set for linoleic acid: an Adequate Intake (AI) and an Acceptable Macronutrient Distribution Range (AMDR) (National Health and Medical Research Council and Ministry of Health, 2006). The AI is the estimated average daily nutrient intake level assumed to be adequate and the AMDR is the range of intake for individuals to reduce chronic disease risk and is based on the highest median intakes for any population group observed in the 1995 NNS.

Age groups used for comparison to these reference health standards in this section differ from those previously presented in this report. Therefore further analysis of the results for linoleic acid based on new population groups was incorporated. The reference health standards are gender split and are presented here for adult males and females separately. Children are grouped together as they have the same reference values.

For all population groups assessed, dietary intakes of linoleic acid at *Baseline* and *Scenario 1* were compared to the AI and AMDR.

Comparison to AI

Given that the AI is an estimate of nutrient intakes by a group of people, it would be expected that around half of each population group would have intakes above this level, and half would have intakes below. At *Baseline*, an average of 65% of respondents from the population groups assessed had intakes at or above the AI for linoleic acid.

For *Scenario 1*, where it is assumed that soybean oil (derived from soybean 305423) has a 25% market share of all edible vegetable oils (except olive), a slightly smaller percentage of respondents from each population group had intakes at or above the AI for linoleic acid. As the AI is established where there insufficient data to determine nutrient requirement distributions and EARs, it is not possible to determine the nutritional impact of a decrease in linoleic acid intakes, as estimated here, over the long term.

See Table 10 for further details.

Table 10: Comparison of *Baseline* and *Scenario 2* dietary intakes of linoleic acid with the AI for various population groups

Country	Population Group	Gender	AI (g/day)	Baseline (%)		Scenario 1 (%)	
				Above AI	Below AI	Above AI	Below AI
Australia	4-8 years	Boys/ Girls	8	60	40	57	43
	9-13 years	Males	10	69	31	68	32
		Females	8	67	33	66	34
	14-18 years	Males	12	70	30	69	31
		Females	8	66	34	64	36
	19+	Males	13	50	50	49	51
		Females	8	53	47	51	49
New Zealand	14-18 years	Males	12	77	23	72	23
		Females	8	81	19	80	20
	19+	Males	13	60	40	56	44
		Females	8	64	36	60	40

Comparison to AMDR

The AMDR for linoleic acid is based on intakes to help reduce chronic disease risk, and in particular, coronary heart disease. At *Baseline*, for all population groups assessed, approximately 50% of respondents had dietary intakes of linoleic acid within the AMDR of 4 or 5-10% of their total daily energy (the lower end of 4% of the AMDR was used for comparison).

For *Scenario 1*, a slightly lower percentage of respondents from each population group and country had dietary intakes within their specified AMDR.

See Table 11 and Table 12 for further details.

Table 11: Comparison of *Baseline* dietary intakes of linoleic acid with the AMDR for various population groups (the lower end of 4% of the range was used for comparison)

Country	Population Group	Gender	AMDR Recommended Intake Range (%)*		Baseline (%)		
			Lower End	Upper End	Below Lower End	Within Range	Above Upper End
Australia	4-8 years	Boys/Girls	4 - 5	10	42	46	12
	9-13 years	Males	4 - 5	10	33	50	17
		Females	4 - 5	10	35	49	16
	14-18 years	Males	4 - 5	10	30	50	20
		Females	4 - 5	10	35	46	19
	19+	Males	4 - 5	10	42	45	13
		Females	4 - 5	10	46	40	14
New Zealand	14-18 years	Males	4 - 5	10	24	59	17
		Females	4 - 5	10	25	60	15
	19+	Males	4 - 5	10	35	52	13
		Females	4 - 5	10	36	52	12

* Of total dietary energy

Table 12: Comparison of *Scenario 2* dietary intakes of linoleic acid with the AMDR for various population groups (the lower end of 4% of the range was used for comparison)

Country	Population Group	Gender	AMDR Recommended Intake Range (%)*		Scenario 1 (%)		
			Lower End	Upper End	Below Lower End	Within Range	Above Upper End
Australia	4-8 years	Boys/Girls	4 - 5	10	46	46	8
	9-13 years	Males	4 - 5	10	35	53	12
		Females	4 - 5	10	38	50	12
	14-18 years	Males	4 - 5	10	32	54	14
		Females	4 - 5	10	37	48	15
	19+	Males	4 - 5	10	43	47	10
		Females	4 - 5	10	47	42	11
New Zealand	14-18 years	Males	4 - 5	10	29	60	11
		Females	4 - 5	10	26	62	12
	19+	Males	4 - 5	10	39	51	10
		Females	4 - 5	10	40	52	8

* Of total dietary energy

Comparison of Estimated Dietary Intake with Applicant's Dietary Intake Assessment

The Applicant provided estimates of fatty acid concentrations in oil from soybean 305423 and assessed 2 different scenarios: intake from the total diet from certain fatty acids and intake of fatty acids from soybean oil only. Intake assessments were based on U.S. food consumption data. Baseline intakes of fatty acids were also assessed for each scenario. Eighteen broad food categories including frozen meals, popcorn, soup, cooking fats, snacks, fried foods, breads and meats were assigned fatty acid concentrations and were modelled to predict dietary intakes of the relevant fatty acids.

For FSANZ's assessment, a baseline intake and one market share scenario was assessed which have previously been discussed. The FSANZ assessment differed from that of the Applicant in that FSANZ used different data for food consumption and concentrations of oleic and linoleic acids for a broader range of foods (120 different foods were assigned oleic and linoleic acid concentrations to predict dietary intakes for different population groups in Australia and New Zealand). FSANZ compared *Scenario 1 – 25% market share* with the Applicant's *Intake from Total Diet* assessment. These were most similar for dietary intake comparison purposes as they both:

- assumed only regular soybean oil was replaced with oil from soybean 305423; and
- included a range of other foods in the diet.

Overall, FSANZ's estimates of dietary intake for all population groups assessed did not differ greatly from that of the Applicant's assessments. Both predicted that should soybean oil derived from soybean 305423 enter the Australian and New Zealand food supply, dietary intakes of oleic or linoleic acids were not altered to a great extent (up to 6% increase for oleic acid and up to 10 % decrease for linoleic acid).

For oleic acid, the Applicant's estimated mean dietary intakes were similar for *Baseline* and *Scenario* estimates made by FSANZ. For Australian children 2-6 years (as compared with the Applicant's U.S. children 1-8 years) FSANZ estimated dietary intakes to be slightly lower at both *Baseline and Scenario 1.*

FSANZ's estimates of mean dietary intakes of linoleic acid were higher for both *Baseline* and *Scenario 1* than the Applicant's dietary intakes. The Applicant did not provide data on dietary intakes for children 1-8 years, however, stated that they were similar to intakes of the U.S. population.

The differences in intakes estimated by FSANZ and those of the Applicant can be attributed to different methodologies used for the assessments. This could include the incorporation of a broader range and different types of food in the FSANZ assessment than in the Applicant's intake estimate, the concentrations assigned and the age groups assessed.

A comparison of the Applicant's and FSANZ's dietary intake assessments can be found in Table A4.1 for oleic acid and Table A4.2 for linoleic acid in Appendix 4.

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Appendix 1

How were the Estimated Dietary Intakes Calculated?

The DIAMOND program allows oleic and linoleic acid concentrations to be assigned to food groups.

The intake of oleic and linoleic acid was calculated for each individual in the NNSs using his or her individual food records from the dietary survey. The DIAMOND program multiplies the specified concentration of oleic or linoleic acid by the amount of food that an individual consumed from that group in order to estimate the intake of oleic or linoleic acid from each food. Once this has been completed for all of the foods specified to contain oleic or linoleic acid, the total amount of oleic or linoleic acid consumed from all foods is summed for each individual. Population statistics (mean and high percentile (90th) intakes) are then derived from the individuals' ranked intakes.

Food consumption amounts for each individual take into account where each food in a classification code is consumed alone and as an ingredient in mixed foods. For example, a glass of milk, the milk in a milkshake, sauce or cake are all included in the consumption of milk.

In DIAMOND, all mixed foods have a recipe. Recipes are used to break down mixed foods into their basic components (e.g. pizza will be broken down into bread, tomato paste, cheese, meat etc.). The data for consumption of the basic components are then used in models that assign oleic and linoleic acid permissions to the relevant food codes.

When a food is classified in two food groups (e.g. cheese, not further specified may be entered into the unripened and ripened cheese categories), and these food groups are assigned different oleic or linoleic acid permissions, DIAMOND will assume if the type of cheese is not specified that the food is in the food group with the highest assigned oleic or linoleic acid level to assume a worst case scenario. If the food groups have the same permitted oleic or linoleic acid level, DIAMOND will assume the food is in the food group that appears first, based alpha-numerically on the DIAMOND food code.

In DIAMOND, hydration and raw equivalence factors are applied to some foods to convert the amount of food consumed in the dietary survey to the equivalent amount of the food in the form to which a food chemical concentration is assigned. Factors are only applied to individual foods, and not major food group codes. For example, consumption figures for cordial concentrate syrup are converted into the equivalent quantities of cordial prepared ready to drink.

Percentage contributions of each food group to total estimated intakes are calculated by summing the intakes for a food group from each individual in the population group who consumed a food from that group and dividing this by the sum of the intakes of all individuals from all food groups containing oleic or linoleic acids, and multiplying this by 100.

Appendix 2

Concentration Data used for the Dietary Intake Assessment

Table A2.1: Mean concentrations of oleic acid and linoleic acid used for the dietary intake assessment (except edible vegetable oils and soybean oil)

Food Code	FOODNAME	Oleic Acid (g/kg)	Linoleic Acid (g/kg)
1.1.1	Liquid milk (including buttermilk)	8.8	0.8
1.1.1.1	Liquid milks, reduced fat	3.4	0.3
1.1.1.2	Liquid milk, low fat	0.2	0
1.1.2	Liquid milk products and flavoured liquid milk	7.6	0.7
1.1.2.1	Liquid milk products, reduced fat	3.6	0.3
1.1.2.2	Liquid milk, artificially sweetened	0.2	0
1.1.2.3	Chocolate flavoured milk (dairy & soy)	0	0
1.1.2.4	Coffee flavoured milk (dairy and soy)	0	0
1.1.2.5	Liquid milk products, low fat	0.2	0
1.2.1	Fermented milk and rennetted milk	10.9	0.9
1.2.1.1	Fermented & rennetted milk, reduced and low fat	0.8	0.1
1.2.2	Fermented milk products and rennetted milk product	8.3	0.7
1.2.2.1	Fermented & rennetted milk products, reduced & low fat	0.8	0.1
1.2.2.2	Fermented & rennetted milk & products, artificially sweetened	0.3	0
1.2.2.3	Frozen fermented & rennet milk products	11.5	0.9
1.3	Condensed milk and evaporated milk	19.9	1.7
1.3.1	Condensed & evaporated milks, reduced & low fat	2	0.2
1.4.1	Cream, reduced cream and light cream	90.1	8.6
1.4.1.1	Cream, reduced cream and light cream, reduced fat	38.2	3.6
1.4.2	Cream products (flavoured, whipped, thickened, sour)	80.7	7.2
1.4.2.1	Cream products (flavoured, whipped, thickened, sour), reduced fat	52.2	4.2
1.5	Dried milk, milk powder, cream powder	6.2	0.5
1.5.1	Dried milk, skim milk powder	0.2	0
1.6.1	Unripened cheese	49.1	4
1.6.1.1	Unripened cheese, reduced fat only	19.5	1.4
1.6.2	Ripened cheese	66.3	5.2
1.6.2.2	Ripened cheese, reduced fat	43.4	4
1.6.4	Processed cheese	57.1	4.4
1.6.4.1	Processed cheese, reduced fat only	17.5	1.4
2.1.1	Olive oil	686	88
2.1.2	Animal based edible oils essentially free of water	373.8	20.1
2.2.1.1	Butter	190.4	16
2.2.1.3	Margarine and similar products	244.7	354
2.2.2	Oil emulsions (< 80 % oil)	326.2	86.8
2.2.2.1	Oil emulsions, reduced fat	119.3	179.8
3.1	Ice cream	20.2	5.3
3.1.1	Ice cream, reduced & low fat	6.4	0.7
3.1.1.2	Choc flavoured & choc chip ice cream (tub style)	0	0
3.1.1.3	Coffee flavoured ice cream (tub style)	0	0

Food Code	FOODNAME	Oleic Acid (g/kg)	Linoleic Acid (g/kg)
3.1.1.4	Choc flavoured or coated ice cream (stick or bar)	0	0
3.1.1.5	Choc cheesecakes and bavarians	0	0
3.1.2	Ice confection	6.4	0.7
3.2	Sherbets and sorbets (frozen)	5.1	0.6
4.3.1.1	Desiccated coconut	32.6	5.5
4.3.1.3	Nuts and seeds	284.8	213.7
4.3.1.4	Dried vegetables	1.1	2.5
4.3.1.5	Dried legumes	2.4	7.4
4.3.2	Fruit & vegetables in vinegar, oil, brine, alcohol	146.6	19.4
4.3.6.3	Peanut butter	237.2	163.9
4.3.6.5	High fat fruit and vegetable preparations	184.2	166.9
4.3.7	Fermented fruit and vegetable products	10	23.7
4.3.7.2	Soy sauce only	0	0
4.3.8	Other fruit and vegetable based products	39.9	52
4.3.8.3	Soy sausages	39.9	52
4.3.8.4	Soy beverages flavoured	5.8	8
4.3.8.5	Soy beverages plain	8.5	11.6
4.3.8.6	Soy beverages plain, low/reduced fat	5.1	6.1
5.0.2	Milk chocolate block/bars (plain)	85.4	9.6
5.0.3	Dark chocolate block/bars (plain)	90.7	8.7
5.0.4	White chocolate block/bars (plain)	96.2	9.5
5.0.5	Cooking chocolate (cocoa based & compounded)	9.6	2.6
5.0.6	Other choc, coffee or cola confectionery	51.2	5.8
5.1	Chocolate and cocoa products (including carob)	70.5	7.6
5.1.1	Chocolate, mixed (including bars, filled etc)	49.1	8
5.1.3	Cocoa powder	47.3	3.8
6.1	Cereals (whole and broken grains)	1.5	5.8
6.1.1	Oats only	34.1	29.9
6.1.2	Rice only	2.3	1.5
6.1.3	Cooked rice only	2.3	1.5
6.2	Flours, meals and starches	7.6	14.5
6.3	Processed cereal and meal products	5.8	10
6.3.3	Puffed and/or extruded cereals	2.7	6.5
6.4	Flour products (including noodles and pasta)	2.3	0
6.4.1	Hotplate products	7.4	5.1
6.4.2	Pasta only	0.6	2.1
6.4.4	Instant noodles	4.1	1.1
6.4.3	Noodles only	4.1	1.1
7.1.1	Plain breads	10.3	14
7.1.1.1	Plain breads, wholemeal	6.8	10.2
7.1.1.2	Plain bread, grain	7.8	9.4
7.1.1.3	Plain bread, rye	4.5	9
7.1.1.4	Plain bread, white, fibre increased	8.4	11.1
7.1.2	Fancy breads	11.5	6.8
7.2.1	Biscuits	58.1	11
7.2.1.1	Biscuits, savoury	32.2	12.7

Food Code	FOODNAME	Oleic Acid (g/kg)	Linoleic Acid (g/kg)
7.2.2	Cakes & muffins	42.5	12.5
7.2.3	Slices	50.3	11.7
7.2.4	Pastries	48.2	8.1
8.1.1	Fresh poultry	35.6	12.4
8.1.3	Veal cuts	6	0.9
8.1.4	Lamb cuts	45	4.8
8.1.5	Rabbit cuts	10.8	6.8
8.1.6	Kangaroo cuts	6.4	1.9
8.1.7	Beef & venison cuts	31.8	3.1
8.1.8	Pork cuts	33.8	9.1
8.2	Ham	26.2	5
8.2.5	Whole pieces of processed meat	27.9	4
8.3	Processed comminuted meat, poultry & game products	69.2	12.2
8.3.1	Fermented, uncooked processed meat products	173.6	29.6
8.3.2	Sausage and sausage meat containing raw, unprocessed meat	80.9	9.4
8.3.3	Frankfurts	87.5	12.1
9.1	Uncooked fish & fish fillets (incl frozen & thawed)	1.1	0.1
9.1.3	Uncooked molluscs	0.8	0.4
9.1.4	Roe	14.4	4.5
9.2	Cooked/processed fish and fish products	3.6	1.3
9.2.1	Cooked/processed crustacea	0.8	0.1
9.2.3	Cooked/processed molluscs	0.2	0.1
9.2.4	Cooked/processed fish only	12.3	2.2
9.3	Semi preserved fish and fish products	9.1	3.5

Appendix 3 – Complete Information from Dietary Intake Assessment

Table A3.1: Estimated dietary intakes for consumers of oleic acid for Australian and New Zealand population groups

Country	Population Group	Scenario	Estimated Dietary Intake of Oleic Acid (g/day)	
			Mean for consumers	90th percentile
Australia	2-6 yrs	Baseline	16.7	26.6
		Scenario 1	17.5	29.1
	2 years & above	Baseline	22.6	40.9
		Scenario 1	23.5	43.3
New Zealand	15 years & above	Baseline	25.3	44.9
		Scenario 1	26.9	48.3

Note:

1. Scenario 1 – 25% market share model assuming soybean oil (derived from soybean 305423) has a 25% market share of the edible vegetable oils (excluding olive oil) market in both Australia and New Zealand.
2. Virtually all respondents were consumers of oleic acid; therefore only consumer dietary intakes have been presented.
3. Total number of respondents for Australia: population aged 2 years and above = 13,858, 2-6 years = 989; New Zealand: population aged 15 years and above = 4,636.

Table A3. 2: Estimated dietary intakes for consumers of linoleic acid for Australian and New Zealand population groups

Country	Population Group	Scenario	Estimated Dietary Exposure to Linoleic Acid (g/day)	
			Mean for consumers	90th percentile
Australia	2-6 yrs	Baseline	10.6	21.0
		Scenario 1	9.7	19.1
	2 years & above	Baseline	14.6	30.6
		Scenario 1	13.6	27.7
New Zealand	15 years & above	Baseline	16.4	32.0
		Scenario 1	14.7	28.2

Note:

1. Scenario 1 – 25% market share model assuming soybean oil (derived from soybean 305423) has a 25% market share of the vegetable oils (excluding olive oil) market in both Australia and New Zealand.
2. Virtually all respondents were consumers of oleic acid; therefore only consumer dietary intakes have been presented.
3. Total number of respondents for Australia: population aged 2 years and above = 13,858, 2-6 years = 989; New Zealand: population aged 15 years and above = 4,636.

Appendix 4 – Comparison Between the Applicant's and FSANZ's Estimated Dietary Intakes

Table A4.1: Estimated dietary intakes of oleic acid – a comparison of the Applicant's and FSANZ's estimates

Data Source	Population Group	Estimated Mean Dietary Intakes of Oleic Acid (g/day)	
		Baseline	Scenario
Applicant	US population	23.4	23.4
FSANZ	Australian population aged 2 years & above	22.6	23.5
FSANZ	New Zealand population aged 15 years & above	25.3	26.9
Applicant	US children aged 1-8 years	18.3	20
FSANZ	Australian children aged 2-6 years	16.7	17.5

Notes:
1. The FSANZ assessment included a broader range of foods than the assessment conducted by the Applicant
2. For the Scenario comparison, FSANZ's *Scenario 1 – 25% market share* was compared with the Applicant's *Intake from Total Diet* assessment

Table A4.2: Estimated dietary intakes of linoleic acid – a comparison of the Applicant's and FSANZ's estimates

Data Source	Population Group	Estimated Mean Dietary Intakes of Linoleic Acid (g/day)	
		Baseline	Scenario
Applicant	US population	10.1	9.1
FSANZ	Australian population aged 2 years & above	14.6	13.6
FSANZ	New Zealand population aged 15 years & above	16.4	14.7

Notes:
1. The FSANZ assessment included a broader range of foods than the assessment conducted by the Applicant
2. For the Scenario comparison, FSANZ's *Scenario 1 – 25% market share* was compared with the Applicant's *Intake from Total Diet* assessment