Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

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Final health and environmental risk assessment of genetically modified soybean 40-3-2

Scientific opinion of herbicide tolerant genetically modified soybean 40-3-2 from Monsanto for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (Application EFSA/GMO/RX/40-3-2)

Opinion of the Panel on Genetically Modified Organisms of the Norwegian Scientific Committee for Food Safety

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2014: 16
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Norwegian Scientific Committee for Food Safety (VKM)
Po 4404 Nydalen
N – 0403 Oslo
Norway

Phone: +47 21 62 28 00
Email: vkm@vkm.no

www.vkm.no
www.english.vkm.no

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Food/feed and environmental risk assessment of herbicide tolerant genetically modified soybean 40-3-2 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (EFSA/GMO/RX/40-3-2)

Authors preparing the draft opinion

Åshild Kristine Andreassen (Chair), Anne Marie Bakke, Knut Helkås Dahl, Hilde-Gunn Hoen-Sorteberg, Monica Sanden, Arne Mikalsen (VKM staff), Ville Erling Sipinen (VKM staff), Rose Vikse (VKM staff) and Merethe Aasmo Finne (VKM staff)

Assessed and approved

The opinion has been assessed and approved by the Panel on Genetically Modified Organisms. Members of the panel are: Åshild Andreassen (chair), Per Brandtzæg, Knut Dahl, Knut Tomas Dalen, Hilde-Gunn Hoen-Sorteberg, Olavi Junttila, Richard Meadow, Kåre M. Nielsen and Monica Sanden

Acknowledgment

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed the Panel on Genetically Modified Organisms (GMO) to answer the request from the Norwegian Food Safety Authority and the Norwegian Environment Agency. Project leaders from the VKM secretariat have been Arne Mikalsen, Ville Erling Sipinen, Rose Vikse and Merethe Aasmo Finne.

Anne Marie Bakke, the Norwegian University of Life Sciences, NMBU and member of the Standing Working Group on Food/Feed Risk Assessment of the VKM GMO Panel, Dagrunn Engeset (VKM staff) and Inger Therese Lillegaard (VKM staff) are acknowledged for their valuable work on this scientific opinion [Chapter 4].

Competence of VKM experts

Experts working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

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Abstract

Soybean 40-3-2 expresses the *cp4 epsps* gene from the common soil bacterium *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) sp. strain CP4. The gene encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), which allows the soybean to tolerate high levels of glyphosate-based herbicides. Updated bioinformatic analyses of the inserted DNA and flanking sequences in soybean 40-3-2 have not indicated a potential production of putative harmful proteins or polypeptides caused by the genetic modification. Genomic stability of the functional insert and consistent expression of the *cp4 epsps* gene has been shown over several generations of soybean 40-3-2. Compositional and agronomic data from numerous field trials performed in North-America and Europe show that soybean 40-3-2 is compositionally, morphologically and agronomically equivalent to its conventional counterpart and to other commercial soybean varieties. Subchronic feeding studies on rats, as well as whole food feeding studies on broilers, catfish, dairy cows, pigs and quail have not indicated any adverse effects of soybean 40-3-2, and indicate that soybean 40-3-2 is nutritionally equivalent to, and as safe as conventional soybean varieties. The CP4 EPSPS protein produced in soybean 40-3-2 does not show sequence resemblance to known toxins or IgE allergens, nor has it been reported to cause IgE-mediated allergic reactions. Soybean is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of soybean in Europe. Based on current knowledge the VKM GMO Panel concludes that with the intended usage, there are no safety concerns associated with soybean 40-3-2 regarding human or animal health or to the environment.

Summary

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Scientific Committee for Food Safety (VKM) has been requested by the Norwegian Environment Agency (former Norwegian Directorate for Nature Management) and the Norwegian Food Safety Authority (NFSA) to conduct final food, feed and environmental risk assessments of all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorised in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC. The request covers scope(s) relevant to the Gene Technology Act. The request does not cover GMOs that VKM already has conducted its final risk assessments on. However, the Agency and NFSA requests VKM to consider whether updates or other changes to earlier submitted assessments are necessary.

The herbicide-tolerant genetically modified soybean 40-3-2 (Unique Identifier MON-Ø4Ø32-6) was approved for import, processing, foods and feeds according to Directive 90/220/EEC in April 1996 (C/UK/94/M3/1). The time period for approval of soybean 40-3-2 expired on 18 April 2007, and a renewal of the authorisation for continued marketing of food and feed containing, consisting of, or produced from soybean 40-3-2 under Regulation (EC) No 1829/2003 was granted on 10 February 2012 (Application EFSA/GMO/RX/40-3-2, Commission Decision 2012/82/EU). An application for cultivation of soybean 40-3-2 in the EU was submitted by Monsanto in November 2005 (EFSA/GMO/NL/2005/24).

Soybean 40-3-2 has previously been assessed as food and feed by the VKM GMO Panel commissioned by the Norwegian Food Safety Authority in connection with the national finalisation of the procedure of the notification C/UK/94/M3/1 in 2007 (VKM 2007). Soybean 40-3-2 has also been evaluated by the VKM GMO Panel as a component of the stacked GM event 305423 x 40-3-2 under Regulation (EC) 1829/2003 in 2009 (Application EFSA/GMO/NL/2007/47) (VKM 2009).

The food, feed and environmental risk assessment of the soybean 40-3-2 is based on information provided by the applicant in the applications EFSA/GMO/RX/40-3-20, EFSA/GMO/NL/2005/24 and C/UK/94/M3/1, and scientific comments from EFSA and other member states made available on the EFSA website GMO Extranet. The risk assessment also considered other relevant peer-reviewed scientific literature.

The VKM GMO Panel has evaluated 40-3-2 with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed. The Norwegian Scientific Committee for Food Safety has also decided to take account of the appropriate principles described in the EFSA guidelines

for the risk assessment of GM plants and derived food and feed (EFSA 2011a), the environmental risk assessment of GM plants (EFSA 2010a), selection of comparators for the risk assessment of GM plants (EFSA 2011b) and for the post-market environmental monitoring of GM plants (EFSA 2011c).

The scientific risk assessment of soybean 40-3-2 include molecular characterisation of the inserted DNA and expression of novel proteins, comparative assessment of agronomic and phenotypic characteristics, nutritional assessments, toxicology and allergenicity, unintended effects on plant fitness, potential for gene transfer, interactions between the GM plant, target and non-target organisms, and effects on biogeochemical processes.

It is emphasised that the VKM mandate does not include assessments of contribution to sustainable development, societal utility and ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These considerations are therefore not part of the risk assessment provided by the VKM Panel on Genetically Modified Organisms. Likewise, the VKM mandate does not include evaluations of herbicide residues in food and feed from genetically modified plants.

Soybean 40-3-2 expresses the gene encoding the enzyme CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS), which is derived from the CP4 strain of *Agrobacterium tumefaciens* (updated scientific name: *Rhizobium radiobacter*), and renders soybean 40-3-2 tolerant to the herbicidal active substance glyphosate.

Molecular characterisation

The applicant has provided sufficient analyses to characterise the DNA inserts, number of inserts, integration sites and flanking sequences in the soybean genome. The results show that one functional copy of the *cp4 epsps* gene only, is present in the soybean 40-3-2 genome. No other functional vector genes were found. Updated similarity searches in 2010, with databases of known toxins and allergens did not indicate a potential production of harmful proteins or polypeptides caused by the genetic modification. Southern blot analyses, segregation studies and phenotypic characteristics of soybean 40-3-2, show that the introduced gene is stably inherited and expressed over multiple generations. The VKM GMO Panel concludes that the molecular characterisation of soybean 40-3-2 does not indicate a safety concern.

Comparative assessment

The VKM GMO Panel has considered the available literature on compositional data and found no biologically significant differences between soybean 40-3-2 and the conventional non-GM control, except small intermittent variations. The data presented do not show unintended effects as a result of the genetic modification. The VKM GMO Panel concludes that soybean

40-3-2 is compositional, agronomical and phenotypically equivalent to its conventional counterpart, and other conventional soybean varieties.

Food and feed risk assessment

Subchronic feeding studies on the glyphosate-tolerant soybean 40-3-2 in rats, as well as whole food feeding studies on broilers, quails, cows, pigs, piglets, catfish and Atlantic salmon have not indicated any adverse effects. The CP4 EPSPS protein does not show sequence resemblance to known toxins or IgE allergens, nor has it been reported to cause IgE mediated allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that soybean 40-3-2 is nutritionally equivalent and as safe as conventional soybean.

Environmental assessment

Considering the intended uses of soybean 40-3-2, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure, mainly through manure and faeces from animals fed grains from soybean 40-3-2.

Soybean 40-3-2 has no altered survival, multiplication or dissemination characteristics compared to conventional soybean, and there are no indications of an increased likelihood of spread and establishment of feral soybean plants in the case of accidental release into the environment of seeds from soybean 40-3-2. Soybean is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of soybean in Europe. Plant to plant gene flow are therefore not considered to be an issue. Considering the intended use as food and feed, interactions with the biotic and abiotic environment are not considered to be an issue.

Overall conclusion

Based on current knowledge, the VKM GMO Panel concludes that soybean 40-3-2 is as safe as its conventional counterpart and as commercial soybean varieties with the intended usage. Soybean 40-3-2 is nutritionally, phenotypically and agronomically equivalent to conventional soybean varieties.

Likewise, the VKM GMO Panel concludes that soybean 40-3-2, based on current knowledge, does not represent an environmental risk in Norway with the intended usage.

Key words: GMO, soybean (Glycine max), 40-3-2, EFSA/GMO/RX/40-3-2, herbicide tolerance, *cp4 epsps,* food and feed safety, environmental risk, Regulation (EC) No 1829/2003, VKM, risk assessment, Norwegian Scientific Committee for Food Safety, Norwegian Environment Agency

Sammendrag på norsk

Som en del av forberedelsene til implementering av EU-forordning 1829/2003 i norsk rett, er Vitenskapskomiteen for mattrygghet (VKM) bedt av Miljødirektoratet (tidligere Direktoratet for naturforvalting (DN)) og Mattilsynet om å utarbeide endelige helse- og miljørisikovurderinger av alle genmodifiserte organismer (GMOer) og avledete produkter som inneholder eller består av GMOer som er godkjent under forordning 1829/2003 eller direktiv 2001/18, og som er godkjent for ett eller flere bruksområder som omfattes av genteknologiloven. Miljødirektoratet og Mattilsynet har bedt VKM om endelige risikovurderinger for de EU-godkjente søknader hvor VKM ikke har avgitt endelige risikovurderinger. I tillegg er VKM bedt om å vurdere hvorvidt det er nødvendig med oppdatering eller annen endring av de endelige helse- og miljørisikovurderingene som VKM tidligere har levert.

Den genmodifiserte, herbicidtolerante soyalinjen 40-3-2 (unik kode MON-Ø4Ø32-6) fra Monsanto ble første gang godkjent til import, videreforedling og til bruk som mat og fôr under EUs tidligere utsettingsdirektiv 90/220/EEC i april 1996 (notifisering C/UK/94/M3/1). Godkjenningen utløp 18. April 2007, og en fornyet godkjenning av mat- og fôrprodukter som inneholder, består av eller er produsert fra soyalinjen ble gitt 10. februar 2012 (fornyingssøknad EFSA/GMO/RX/40-3-2, Kommisjonsbeslutning 2012/82/EU). En søknad om godkjenning av soya 40-3-2 til dyrking ble fremmet av Monsanto i november 2005 under forordning 1829/2003/EF (EFSA/GMO/NL/2005/24).

Soyalinjen 40-3-2 ble første gang vurdert av VKMs faggruppe for GMO i 2007 (VKM 2007). Helserisikovurderingen ble utført på oppdrag av Mattilsynet i forbindelse med vurdering av markedsadgang i Norge. VKMs faggruppe for GMO har også risikovurdert en soyahybrid der den genmodifiserte soyaen inngår som en av foreldrelinjene (EFSA/GMO/NL/2007/47 - 305423 x 40-3-2 (VKM 2009).

Risikovurderingen av den genmodifiserte soyalinjen er basert på uavhengige vitenskapelige publikasjoner og dokumentasjon som er gjort tilgjengelig på EFSAs nettside EFSA GMO Extranet. Vurderingen er gjort i henhold til tiltenkt bruk i EU/EØS-området, og i overensstemmelse med miljøkravene i genteknologiloven med forskrifter, først og fremst forskrift om konsekvensutredning etter genteknologiloven. Videre er kravene i EU-forordning 1829/2003/EF, utsettingsdirektiv 2001/18/EF (vedlegg 2,3 og 3B) og veiledende notat til Annex II (2002/623/EF), samt prinsippene i EFSAs retningslinjer for risikovurdering av genmodifiserte planter og avledete næringsmidler (EFSA 2006; 2010; 2011 a,b,c) lagt til grunn for vurderingen.

Den vitenskapelige vurderingen omfatter transformeringsprosess og vektorkonstruksjon, karakterisering og nedarving av genkonstruksjonen, komparativ analyse av ernæringsmessig kvalitet, mineraler, kritiske toksiner, metabolitter, antinæringsstoffer, allergener og nye

proteiner. Videre er agronomiske egenskaper, potensiale for utilsiktede effekter på fitness, genoverføring, og effekter på målorganismer, ikke-målorganismer og biogeokjemiske prosesser vurdert.

Det presiseres at VKMs mandat ikke omfatter vurderinger av etikk, bærekraft og samfunnsnytte, i henhold til kravene i den norske genteknologiloven og dens konsekvensutredningsforskrift. Disse aspektene blir derfor ikke vurdert av VKMs faggruppe for genmodifiserte organismer. Vurderinger av mulige plantevernmiddelrester i den genmodifiserte planten som følge av endret sprøytemiddelbruk faller per i dag utenfor VKMs ansvarsområde og er derfor heller ikke vurdert.

Soya 40-3-2 uttrykker *cp4 epsps*-genet fra jordbakterien *Agrobacterium tumefaciens* (syn. *Rhizobium radiobacter*). Genet koder for enzymet 5-enolpyruvylsikimat-3-fosfatsyntetase, som omdanner fosfoenolpyruvat og sikimat-3-fosfat til 5-enolpyruvylsikimat-3-fosfat, en viktig metabolitt i syntesen av aromatiske aminosyrer. I motsetning til plantens eget enzym er det bakterielle enzymet også aktivt ved nærvær av N-fosfonometylglycin (glyfosat). De transgene plantene vil derfor tolerere høyere doser av herbicider med virkestoffet glyfosat sammenlignet med konkurrerende ugras.

Molekylær karakterisering

Søkeren har oppgitt tilstrekkelige analysedata til å karakterisere de introduserte DNA-innskuddene, antallet integreringer, integreringssteder, og innskuddenes flankerende DNA-sekvenser i genomet til soya 40-3-2. Resultatene viser at kun ett funksjonelt *cp4 epsps* gen er integrert i genomet til soyalinjen. Oppdaterte homologisøk fra 2010 med databaser over kjente toksiner og allergener indikerer at genmodifiseringen ikke har ført til potensiell produksjon av skadelige proteiner eller polypeptider i soya 40-3-2. Southern blot og segresjons -analyser viser at det introduserte genet er stabilt nedarvet og uttrykt over flere generasjoner, og i samsvar med de fenotypiske egenskapene til soya 40-3-2. VKMs faggruppe for GMO konkluderer med at den molekylære karakteriseringen ikke indikerer noen helserisiko ved soya 40-3-2.

Komparative analyser

VKMs faggruppe for GMO har vurdert tilgjengelig litteratur vedrørende soya 40-3-2 og konkludert at soyaen er ernæringsmessig, morfologisk og agronomisk vesentlig lik dens konvensjonelle motpart, samt andre konvensjonelle varianter. Med unntak av enkelte små variasjoner, ble det ikke funnet forskjeller av biologiske betydning mellom den genmodifiserte soyalinjen og dens konvensjonelle kontroll. Forøvrig viser de rapporterte dataene ingen utilsiktede effekter som følge av genmodifiseringen.

Helserisiko

Subkroniske fôringsstudier utført på rotter, samt helmatsstudier på broiler, vaktler, kyr, gris, pattegris, malle og atlanterhavslaks har ikke indikert helseskadelige effekter av soya 40-3-2. CP4 EPSPS-proteinet viser ingen likhetstrekk til kjente toksiner eller allergener, og er heller ikke rapporterte å ha forårsaket IgE-medierte allergiske reaksjoner. Det er usannsynlig at CP4 EPSPS-proteinet vil resultere i en toksisk eller allergen effekt.

Ut i fra dagens kunnskap konkluderer VKMs faggruppe for GMO at soya 40-3-2 er ernæringsmessig lik og like trygg som konvensjonell soya.

Miljørisiko

Med bakgrunn i tiltenkt bruksområde for søknaden er miljørisikovurderingen av soyalinje 40-3-2 avgrenset til mulige effekter av utilsiktet spredning av spiredyktige frø i forbindelse med transport og prosessering, samt indirekte eksponering gjennom gjødsel fra husdyr fôret med genmodifisert soya. Faggruppen har ikke vurdert mulige miljøeffekter knyttet til dyrking av soyalinjen.

Genmodifiseringen av soya 40-3-2 har ikke medført endringer i egenskaper knyttet til overlevelse, oppformering eller spredning sammenlignet med konvensjonell soya, og det er ingen indikasjoner på økt sannsynlighet for spredning og etablering av ferale soyaplanter fra utilsiktet frøspill av soyalinjen. Soya dyrkes ikke i Norge, og arten har ikke viltvoksende populasjoner eller nærstående arter utenfor dyrking i Europa. Det er derfor ikke risiko for utkryssing med dyrkede sorter eller ville planter i Norge.

Samlet vurdering

Ut i fra dagens kunnskap konkluderer VKMs faggruppe for GMO at soya 40-3-2, ved forskreven bruk, er like trygg som dens konvensjonelle motpart og annen kommersiell soya. Soya 40-3-2 er ernæringsmessig, morfologisk og agronomisk ekvivalent med konvensjonell soya.

Likeledes finner faggruppen, ut i fra dagens kunnskap, at den omsøkte bruken av soya 40-3-2 ikke vil medføre noen miljørisiko i Norge.

Abbreviations and explanations

ARMG	Antibiotic resistance marker gene
BC	Backcross. Backcross breeding in maize is extensively used to move a single trait of interest (e.g. disease resistance gene) from a donor line into the genome of a preferred or "elite" line without losing any part of the preferred lines existing genome. The plant with the gene of interest is the donor parent, while the elite line is the recurrent parent. BC_1, BC_2 etc. designates the backcross generation number.
BLAST	Basic Local Alignment Search Tool. Software that is used to compare nucleotide (BLASTn) or protein (BLASTp) sequences to sequence databases and calculate the statistical significance of matches, or to find potential translations of an unknown nucleotide sequence (BLASTx). BLAST can be used to understand functional and evolutionary relationships between sequences and help identify members of gene families.
bp	Basepair
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower mosaic virus
Codex	Set by The Codex Alimentarius Commission (CAC), an intergovernmental body to implement the Joint FAO/WHO Food Standards Programme. Its principle objective is to protect the health of consumers and to facilitate the trade of food by setting international standards on foods (i.e. Codex Standards).
Cp4 epsps	Gene from *Agrobacterium tumefaciens* strain CP4
CTP	Chloroplast transit peptide
DAP	Days after planting
DNA	Deoxyribonucleic acid
DT50	Time to 50% dissipation of a protein in soil
DT90	Time to 90% dissipation of a protein in soil
dw	Dry weight
dwt	Dry weight tissue
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
ERA	Environmental risk assessment
E-score	Expectation score
EU	European Union
fa	Fatty acid
FAO	Food and Agriculture Organisation
FIFRA	US EPA Federal Insecticide, Fungicide and Rodenticide Act

Fitness	Describes an individual's ability to reproduce successfully relative to that of other members of its population.
fw	Fresh weight
fwt	Fresh weight tissue
GAT	Glyphosate N-acetyltransferase
GLP	Good Laboratory Practice
Glyphosate	Broad-spectrum systemic herbicide
GM	Genetically Modified
GMO	Genetically Modified Organism
GMP	Genetically Modified Plant
H	Hybrid
ha	Hectare
ILSI	International Life Sciences Institute
IPM	Integrated Pest Management
IRM	Insect Resistance Management
Locus	The position/area that a given gene occupies on a chromosome
LOD	Limit of detection
LOQ	Limit of quantification
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight. A mass spectrometry method used for detection and characterisation of biomolecules, such as proteins, peptides, oligosaccharides and oligonucleotides, with molecular masses between 400 and 350,000 Da.
mRNA	Messenger RNA
MT	Norwegian Food Safety Authority (Mattilsynet)
NDF	Neutral detergent fibre, measure of fibre used for animal feed analysis. NDF measures most of the structural components in plant cells (i.e. lignin, hemicellulose and cellulose), but not pectin.
Northern blot	Northern blot is a technique used to study gene expression by detection of RNA or mRNA separated in a gel according to size.
NTO	Non-target organism
Nicosulfuron	Herbicide for maize that inhibits the activity of acetolactate synthase
Near-isogenic lines	Term used in genetics/plant breeding, and defined genetic lines that are identical except for differences at a few specific locations or genetic loci.
OECD	Organisation for Economic Co-operation and Development
ORF	Open Reading Frame, in molecular genetics defined as a reading frame that can code for amino acids between two stop codons (without stop codons).
OSL	Over season leaf
OSR	Over season root
OSWP	Over season whole plant
PCR	Polymerase chain reaction, a technique to amplify DNA by copying it

R0	First transformed generation, parent	
RNA	Ribonucleic acid	
RP	Recurrent parent	
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Technique to separate proteins according to their approximate size	
SAS	Statistical Analysis System	
SD	Standard deviation	
Southern blot	Method used for transfer of electrophoresis-separated DNA fragments to a filter membrane and possible subsequent fragment detection by probe hybridisation	
Soybean Growth Stages	**Vegetative Stages**	**Reproductive Stages**
	VE - Emergence	R1 – Beginning flowering
	VC - Cotyledon stage	R2 – Full flowering
	V1- First trifoliolate	R3 – Beginning pod (pods 5 mm in top 4 nodes)
	V2 – Second trifoliolate	R4 – Full pod (pods 2 cm in top 4 nodes)
	V3 – Third trifoliolate	R5 – Beginning seed (seed 3 mm long in top 4 nodes)
	V(n) – nth trifoliolate	R6 – Full size seed (pod containing a green seed that fills the pod capacity in top 4 nodes on the main stem)
		R7 – Beginning maturity (one pod on the main stem has reached its mature pod colour)
		R8 – Full maturity (95 % of the pods on the plant have reach their full mature colour)
T-DNA	Transfer DNA, the transferred DNA of the tumour-inducing (Ti) plasmid of some species of bacteria such as *Agrobacterium tumefaciens* and *A. rhizogenes*, into plant's nuclear genome. The T-DNA is bordered by 25-base-pair repeats on each end. Transfer is initiated at the left border and terminated at the right border and requires the *vir* genes of the Ti plasmid.	
TI	Trait integrated	
TMDI	Theoretical Maximum Daily Intake	
Transgene copy number	Transgene copy number is defined as the number of exogenous DNA insert(s) in the genome. If the exogenous DNA fragment inserts only once at a single locus of the genome, it is a single copy	

	transgenic event.
U.S. EPA	United States Environmental Protection Agency
Western blot	Technique used to transfer proteins separated by gel electrophoresis by 3-D structure or denaturated proteins by the length of the polypeptide to a membrane, where they might be identified by antibody labelling.
WHO	World Health Organisation

Background

The herbicide-tolerant soybean 40-3-2 (Unique Identifier MON-Ø4Ø32-6) was approved for import, processing, foods and feeds on the EU-market according to Directive 90/220/EEC already in April 1996 (Commission Decision 96/281/EC). It is still the major genetically modified soybean grown worldwide. After the new EU-regulations on genetically modified food and feed products (Regulation 1829/2003/EC) came into force in 2004, previously approved GMOs were permitted to remain on the market as "existing products". The time period for approval of soybean 40-3-2 expired on 18 April 2007.

On 29 June 2007, the European Food Safety Authority (EFSA) received from the European Commission two applications for renewal of the authorisation soybean 40-3-2, submitted by Monsanto within the framework of Regulation (EC) No 1829/2003.

The scopes of the renewal applications cover:

- Existing food containing, consisting of, or produced from soybean 40-3-2 (including food additives) (**EFSA-GMO-RX-40-3-2$_{[8\text{-}1a/20\text{-}1a]}$**) that have been placed on the market in accordance with Part C to the Directive 90/220/EC before the entry into force of Regulation (EC) No 258/97 and under Directive 89/107/EEC (Commission Decision 96/281/EC);
- Existing feed containing, consisting of, or produced from soybean 40-3-2 (**EFSA-GMO-RX-40-3-2$_{[8\text{-}1b/20\text{-}1b]}$**) that have been placed on the market in accordance with Part C to the Directive 90/220/EEC (Commission Decision 96/281/EC) and as feed materials and feed additives subject to Directive 70/524/EEC;
- Products other than food and feed containing or consisting of soybean 40-3-2 for the same uses as any other soybean with the exception of cultivation (Commission Decision 96/281/EC).

After the date of entry into force of the Regulation (EC) No 1829/2003, the products mentioned above were notified to the European Commission according to Articles 8 or 20 of this Regulation and included in the Community Register of GM food and feed.

After receiving the renewal applications EFSA-GMO-RX-40-3-2$_{[8\text{-}1a/20\text{-}1a}$ and EFSA-GMO-RX-40-3-2$_{[8\text{-}1b/20\text{-}1b]}$, EFSA informed the EU- and EFTA Member States (MS) and the European Commission and made the summary of the dossiers publicity available on the EFSA website. EFSA initiated a formal review of the applications to check compliance with the requirements laid down in Articles 5(3) and 17(3) of regulation (EC) No 1829/2003. On 3 March 2008, EFSA received additional information requested under completeness check, and on 12 March 2008 EFSA declared the applications as valid in accordance with Articles 6(1) and 18(1) of Regulation (EC) No 1829/2003.

EFSA made the valid application available to Member States and the EC and consulted nominated risk assessment bodies of the MS, including the Competent Authorities within the meaning of Directive 2001/18/EC (EC 2001) to request their scientific opinion. Within three months following the date of validity, all MS could submit via the EFSA GMO Extranet to EFSA comments or questions on the valid applications under assessment.

EFSA published its scientific opinion 10 November 2010 (EFSA 2010b), and the authorisation for continued marketing of products containing, consisting of, or produced from GM soybean 40-3-2 was granted 10 February 2012 (Commission Decision 2012/82/EU).

An application for authorisation of soybean 40-3-2 for cultivation in the EU was submitted by Monsanto in 4 November 2005 (EFSA/GMO/NL/2005/24). The German Competent Authority evaluated the initial environmental risk assessment of the application and after receiving additional information requested under completeness check, EFSA declared the application as valid on 29 December 2006. On 31 May 2012 the EFSA GMO Panel adopted its scientific opinion on soybean 4-3-2 (EFSA 2012).

Soybean 40-3-2 has previously been assessed as food and feed by the VKM GMO Panel commissioned by the Norwegian Food Safety Authority in connection with the national finalisation of the procedure of the notification C/UK/94/M3/1 in 2007 (VKM 2007). Soybean 40-3-2 has also been evaluated by the VKM GMO Panel as a component of the stacked GM event 305423 x 40-3-2 under Regulation (EC) 1829/2003 (VKM 2009).

Exemption of the authorisation requirements of 19 existing products in Norway
Through the Agreement of the European Economic Area (EEA), Norway is obliged to implement the EU regulations on genetically modified (GM) food and feed (regulations 1829/2003, 1830/2003 et al.). Until implementation of these regulations, Norway has a national legislation concerning processed GM food and feed products that are harmonised with the EU legislation. These national regulations entered into force 15 September 2005. For genetically modified feed and some categories of genetically modified food, no requirements of authorisation were required before this date. Such products, lawfully placed on the Norwegian marked before the GM regulations entered into force, the so-called existing products, could be sold in a transitional period of three years when specific notifications were sent to the Norwegian Food Safety Authority. Within three years after 15. September 2005, applications for authorisation had to be sent to the Authority before further marketing.

The Norwegian Seafood Federation (FHL) has once a year since 2008, applied for an exemption of the authorisation requirements of 19 existing GM products. These 19 GM events are all authorised in the EU, and the Norwegian Food Safety Authority (NFSA) has granted exemption for a period of one year at the time.

According to the NFSA, FHL has applied for an exemption in the case of a feed shortage, but no GM ingredients has so far been used by the Norwegian fish feed industry. In October 2014, a new application from the FHL to prolong the exemption was rejected by the NFSA.

Terms of reference

The Norwegian Environment Agency (former Norwegian Directorate for Nature Management) has the overall responsibility for processing applications for the deliberate release of genetically modified organisms (GMOs). This entails inter alia coordinating the approval process, and to make a holistic assessment and recommendation to the Ministry of the Environment regarding the final authorisation process in Norway. The Agency is responsible for assessing environmental risks on the deliberate release of GMOs, and to assess the product's impact on sustainability, benefit to society and ethics under the Gene Technology Act.

The Norwegian Food Safety Authority (NFSA) is responsible for assessing risks to human and animal health on deliberate release of GMOs pursuant to the Gene Technology Act and the Food Safety Act. In addition, the NFSA administers the legislation for processed products derived from GMO and the impact assessment on Norwegian agriculture according to sector legislation.

The Norwegian Environment Agency

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Environment Agency, by letter dated 13 June 2012 (ref. 2008/4367/ART-BI-BRH), requests the Norwegian Scientific Committee for Food Safety, to conduct final environmental risk assessments for all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorised in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC. The request covers scope(s) relevant to the Gene Technology Act. The request does not cover GMOs that the Committee already has conducted its final risk assessments on. However, the Norwegian Environment Agency requests the Committee to consider whether updates or other changes to earlier submitted assessments are necessary.

The basis for evaluating the applicants' environmental risk assessments is embodied in the Act Relating to the Production and Use of Genetically Modified Organisms etc. (the Norwegian Gene Technology Act), Regulations relating to impact assessment pursuant to the Gene Technology Act, the Directive 2001/18/EC on the deliberate release of genetically modified organisms into the environment, Guidance note in Annex II of the Directive 2001/18 (2002/623/EC) and the Regulation 1829/2003/EC. In addition, the EFSA guidance documents on risk assessment of genetically modified plants and food and feed from the GM plants (EFSA 2010a, 2011a), and OECD guidelines will be useful tools in the preparation of the Norwegian risk assessments.

The risk assessments' primary geographical focus should be Norway, and the risk assessments should include the potential environmental risks of the product(s) related to any changes in agricultural practices. The assignment covers assessment of direct environmental

impact of the intended use of pesticides with the GMO under Norwegian conditions, as well as changes to agronomy and possible long-term changes in the use of pesticides.

The Norwegian Food Safety Authority

In preparation for a legal implementation of EU-regulation 1829/2003, the Norwegian Environment Agency has requested the Norwegian Food Safety Authority (NFSA) to give final opinions on all genetically modified organisms (GMOs) and products containing or consisting of GMOs that are authorised in the European Union under Directive 2001/18/EC or Regulation 1829/2003/EC within the Authority's sectoral responsibility. The request covers scope(s) relevant to the Gene Technology Act.

The Norwegian Food Safety Authority has therefore, by letter dated 13 February 2013 (ref. 2012/150202), requested the Norwegian Scientific Committee for Food Safety (VKM) to carry out final scientific risk assessments of 39 GMOs and products containing or consisting of GMOs that are authorised in the European Union. The assignment from NFSA includes food and feed safety assessments of genetically modified organisms and their derivatives, including processed non-germinating products, intended for use as or in food or feed.

In the case of submissions regarding genetically modified plants (GMPs) that are relevant for cultivation in Norway, VKM is also requested to evaluate the potential risks of GMPs to the Norwegian agriculture and/or environment. Depending on the intended use of the GMP(s), the environmental risk assessment should be related to import, transport, refinement, processing and cultivation. If the submission seeks to approve the GMP(s) for cultivation, VKM is requested to evaluate the potential environmental risks of implementing the plant(s) in Norwegian agriculture compared to existing varieties (e.g. consequences of new genetic traits, altered use of pesticides and tillage). The assignment covers both direct and secondary effects of altered cultivating practices.

VKM is further requested to assess risks concerning coexistence of cultivars. The assessment should cover potential gene flow from the GMP(s) to conventional and organic crops as well as to compatible wild relatives in semi-natural or natural habitats. The potential for establishment of volunteer populations within the agricultural production systems should also be considered. VKM is also requested to evaluate relevant segregation measures to secure coexistence during agricultural operations up to harvesting. Post-harvest operations, transport, storage are not included in the assignment.

Evaluations of suggested measures for post-market environmental monitoring provided by the applicant, case-specific monitoring and general surveillance, are not covered by the assignment from the Norwegian Food Safety Authority. In addition, the changes related to herbicide residues of genetically modified plants as a result of the application of plant-protection products fall outside the remit of the Norwegian VKM panels.

Assessment

1 Introduction

Genetically modified soybean 40-3-2 (Unique Identifier MON-Ø4Ø32-6) was developed to provide tolerance to the broad spectrum systemic herbicide glyphosate, the active ingredient in Roundup and other brands, widely used in a variety of weed control programs throughout most of the world. Glyphosate is phytotoxic to the majority of annual and perennial grasses and broadleaved weeds. Its mode of action is to inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an essential enzyme involved in aromatic amino acid synthesis in plants, bacteria and fungi. Blocking of the enzyme results in lack of synthesis of the aromatic amino acids tyrosine, tryptophan and phenylalanine (OECD, 1999). The distribution of this pathway and the resulting inability to produce key amino acids prevents growth and ultimately leads to the death of the plant.

In glyphosate-tolerant soybean 40-3-2, the herbicide tolerance trait is generated in the plants through the addition of a bacterial *epsps* gene derived from a common soil bacterium, *Agrobacterium tumefaciens* (updated scientific name: *Rhizobium radiobacter*) sp. strain CP4 (CP4 EPSPS). The enzyme produced from the *cp4 epsps* gene has a lower affinity to the herbicide compared with the innate soybean enzyme, and thus confers glyphosate-tolerance to the whole plant.

The genetic modification in soybean 40-3-2 is intended to improve agronomic performance only and is not intended to influence the nutritional properties, the processing characteristics and the overall use of soybean as a crop.

Soybean 40-3-2 has been evaluated with reference to its intended uses in the European Economic Area (EEA), and according to the principles described in the Norwegian Food Act, the Norwegian Gene Technology Act and regulations relating to impact assessment pursuant to the Gene Technology Act, Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms, and Regulation (EC) No 1829/2003 on genetically modified food and feed.

The Norwegian Scientific Committee for Food Safety has also taken into account the appropriate principles described in the EFSA guidelines for the risk assessment of GM plants and derived food and feed (EFSA 2011a), the environmental risk assessment of GM plants (EFSA 2010a), the selection of comparators for the risk assessment of GM plants (EFSA 2011b), and for the post-market environmental monitoring of GM plants (EFSA 2011c).

The food, feed and environmental risk assessment of the genetically modified soybean 40-3-2 is based on information provided by the applicant in the applications EFSA/GMO/RX/40-3-

2, EFSA/GMO/NL/2005/24 and the notification C/UK/94/M3/1, relevant peer-reviewed scientific literature, and scientific opinions and comments from EFSA and other member states made available on the EFSA website GMO Extranet.

It is emphasised that the VKM mandate does not include assessments of contribution to sustainable development, societal utility and ethical considerations, according to the Norwegian Gene Technology Act and Regulations relating to impact assessment pursuant to the Gene Technology Act. These considerations are therefore not part of the risk assessment provided by the VKM Panel on Genetically Modified Organisms.

2 Molecular characterisation

2.1 Information related to the genetic modification

2.1.1 Description of the methods used for the genetic modification

Plasmid vector PV-GMGT04 was used to produce the glyphosate tolerant soybean 40-3-2 by the particle acceleration method. DNA fragments with the genes of interest from plasmid PV-GMGT04 were accelerated to penetrate callus cells of the soybean cultivar A5403, and some DNA fragments were incorporated in the targeted calli genomes.

Next the callus cells were incubated on a plant tissue culture medium containing cytokinin and auxin to induce shoot formations. The plasmid vector PV-GMGT04 contains two gene cassettes with *cp4 epsps,* and one with *uidA* (Figure 2.1.2-1). *UidA* encodes the β-D-glucuronidase (GUS) protein from *E.coli,* used to identify transformed shoots (R0-generation). R1 plants were grown in a greenhouse, and sprayed with glyphosate for further selection and assessments, i.e. tolerance to glyphosate and inheritance patterns to identify single copy transgenics. R2 progeny of selected single R1 plants were evaluated for glyphosate tolerance in the field. According to the applicant, field segregation data, combined with testing for GUS activity suggested that the R2 progeny designated soybean 40-3-2 was homozygous for the functional *cp4 epsps* insert regulated by the cauliflower mosaic virus promoter (P-E35S in Figure 2.1.2-1). The results also suggested that the soybean lacked an active *uidA,* and the other functional *cp4 epsps* insert regulated by the modified figwort mosaic virus promoter (P-FMV in Figure 2.1.2-1), (Padgette et al., 1995). These results were later confirmed by molecular analyses. A flow chart for the development of soybean 40-3-2 is shown in Figure AI-1 (Appendix I).

2.1.2 Nature and source of vector used

The vector PV-GMGT04 (Figure 2.1.2-1) is a derivative of the high copy *E. coli* plasmid pUC119 (Vieira and Messing., 1987). Table 2.1.2-1 lists the genetic elements present in PV-GMGT04. A schematic diagram of the insert in soybean 40-3-2 is shown in Figure 2.1.2-2.

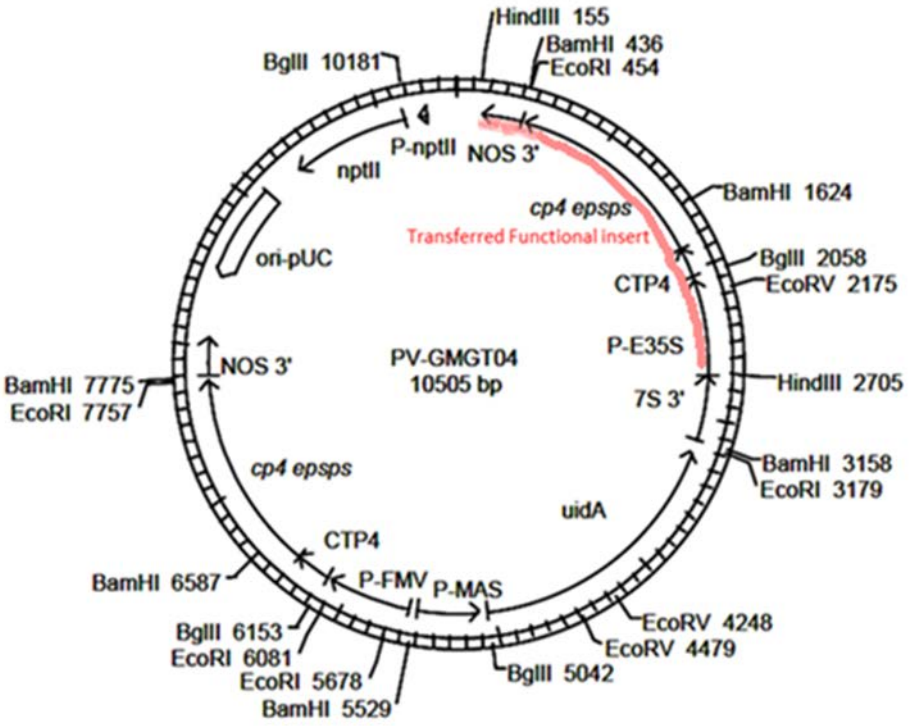

Figure 2.1.2-1. Plasmid map of vector PV-GMGT04. Genetic elements are annotated in the interior of the map and restriction sites (with positions relative to the site of the plasmid vector) are shown for enzymes used in the Southern analyses on the exterior (Adapted from Figure 1 in Technical dossier).

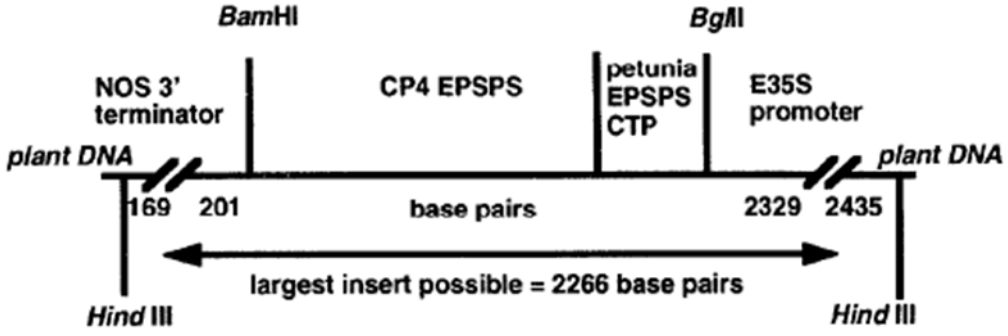

Figure 2.1.2-2. Schematic diagram of the DNA insert in soybean 40-3-2 (Padgette et al. 1995)

Table 2.1.2-1. Summary of DNA components of the plasmid vector PV-GMGT04 (Table 3 in Technical dossier).

Genetic elements	Size (kb)	Function
P-E35S	0.61	The cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al.*, 1985) with the duplicated enhancer region (Kay *et al.*, 1987).
CTP4	0.22	The N-terminal chloroplast transit peptide sequence from the *Petunia hybrida epsps* gene (Shah *et al.*, 1986).
cp4 epsps	1.36	The 5-enolpyruvylskikimate-3-phosphate synthase coding sequence (*cp4 epsps*) from *Agrobacterium* sp. strain CP4 (Barry *et al.*, 1992).
NOS 3'	0.26	The 3' non-translated region of the nopaline synthase gene, which directs polyadenylation of the mRNA (Fraley *et al.*, 1983).
nptII	1.32	The gene for the enzyme neomycin phosphotransferase type II from Tn5, a transposon isolated from *E. coli* (Beck *et al.*, 1982), which allows selection of bacteria containing the plasmid.
ori-pUC	0.65	The origin of replication from the high copy *E. coli* plasmid pUC119 (Vieira and Messing, 1987), necessary for replicating the plasmid in *E. coli*.
P-MAS	0.42	The TR 2' mannopine synthase promoter region (Velten, 1984).
uidA	1.81	The *uidA* coding sequence from *E. coli* encoding a ß-D-glucuronidase (GUS) protein gene (Jefferson *et al.*, 1986).
7S 3'	0.43	The 3' nontranslated region of the alpha subunit of the soybean 7S seed storage protein complex (Schuler *et al.*, 1982).
P-FMV	0.57	The 35S promoter from a modified figwort mosaic virus (FMV) (Gowda *et al.*, 1989; Richins *et al.*, 1987; Sanger *et al.*, 1990; Shepherd *et al.*, 1987).
CTP4	0.22	The N-terminal chloroplast transit peptide sequence from the *Petunia hybrida epsps* gene (Shah *et al.*, 1986).
cp4 epsps	1.36	The 5-enolpyruvylskikimate-3-phosphate synthase coding sequence (*cp4 epsps*) from *Agrobacterium* sp. strain CP4 (Barry *et al.*, 1992).
NOS 3'	0.26	The 3' non-translated region of the nopaline synthase gene, which directs polyadenylation of the mRNA (Fraley *et al.*, 1983).

2.1.3 Source of donor DNA, size and intended function of each constituent fragment of the region intended for insertion

Plasmid PV-GMGT04 contains three gene cassettes intended for insertion, of which two contain the *cp4 epsps* coding sequence and one contains the *uidA* coding sequence (Table 2.1.2-1). In both *cp4 epsps* gene cassettes, the *cp4 epsps* coding sequence is linked to a chloroplast transit peptide sequence designated CTP4, based on the CTP sequence isolated from the Petunia hybrida 5-enolpyruvylshikimate-3-phosphate synthase (*epsps*) gene. CTP4 targets the CP4 EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid biosynthesis. The two *ctp4-cp4 epsps* coding sequences of the two gene cassettes in plasmid PV-GMGT04 are controlled by the enhanced 35S cauliflower mosaic virus promoter (P-E35S) and the 35S figwort mosaic virus promoter (P-FMV), respectively (Figure 2.1.2-1). Both promoters are considered constitutively active in plants.

In both *cp4 epsps* gene cassettes, the *cp4 epsps* coding sequence is joined to the nopaline synthase 3' non-translated sequence (NOS 3') from *Agrobacterium*, which provides the polyadenylation sites directing mRNA processing. The *cp4 epsps* coding sequence, isolated from *Agrobacterium* sp. strain CP4, encodes the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) which confers a high level of tolerance to glyphosate, unlike most native plant and microbial EPSPS enzymes. EPSPS catalyses the conversion of shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) into 5-enolpyruvylshikimate-3-phophate (EPSP), an intermediate required for the production of aromatic amino acids. The *uidA* gene cassette contains the *uidA* coding sequence under the control of the TR 2' mannopine synthase promoter and is joined to the 7S 3' non-translated region of the alpha subunit of the soybean 7S seed storage protein complex. The *uidA* coding sequence originates from *E. coli*. The GUS protein encoded by the *uidA* gene cassette is a 68 kD acid hydrolase that catalyses the cleavage of several β-glucuronides and has been used as a scoreable marker in the transformation and regeneration of soybean 40-3-2. The DNA inserted in soybean 40-3-2 is limited to a single functional gene cassette (the *cp4 epsps* driven by P-E35S). The *uidA* and *cp4 epsps* gene cassettes driven by P-FMV, and all other elements of the plasmid vector are not present in the genome of soybean 40-3-2.

2.2 Information relating to the GM plant

2.2.1 Description of the trait(s) and characteristics which have been introduced or modified

Soybean 40-3-2 contains a gene encoding the CP4 EPSPS protein, which confers tolerance to glyphosate.

2.2.2 Information on the sequences actually inserted or deleted

2.2.2.1 The size and copy number of all detectable inserts, both complete and partial

Molecular analyses were conducted to characterise the DNA insert in soybean 40-3-2. Genomic DNA was analysed by Southern blot to determine the insert number (number of integration sites of the transgene within the soybean genome) and copy number (number of repeats/copies of the transgene sequence within one integration site). To further define the insert and to determine the integrity of the inserted promoters, coding sequences and polyadenylation sequences and the presence or absence of any other elements of plasmid vector PV-GMGT04, a combination of Southern blot, PCR and genome walking analyses was used.

2.2.2.2 Southern blot analyses to determine insert and copy number

In order to determine the insert and copy number in soybean 40-3-2, Southern blot analyses were conducted on isolated genomic DNA of soybean 40-3-2 and the control A5403 with three restriction enzymes that cut within plasmid PV-GMGT04 (*BamHI, HindIII* and *EcoRI*) (Figure 2.2.2.2-1) and a 32P-labelled PV-GMGT04 probe. In the *BamHI* digestion of soybean 40-3-2, a 1.2 kb fragment corresponded to the ~1.2 kb fragment of PV-GMGT04 (1624bp – 436bp = 1188bp, Figure 2.1.2-1 and Table 2.2.2.2-1). Two additional hybridising bands of 2900 and 350 bp, which did not match in size to any band in the *BamHI* PV-GMGT04 digest, were border fragments that contain part of the plasmid DNA attached to plant genomic DNA. *HindIII* cuts twice within PV-GMGT04 (see Figure 2.1.2-1 and Table 2.2.2.2-1), but only one hybridising band of approximately 5.8 kb was clearly detected in soybean 40-3-2, indicating that at least one *HindIII* site was absent in the insert. Sequence analyses later confirmed the absence of both *HindIII* sites in soybean 40-3-2. An *EcoRI* site is present in PV-GMGT04 near the 3' end of the *cp4 epsps* coding sequence (Figure 2.1.2-1). Two hybridising bands of 1.9 and 2.9 kb were detected after *EcoRI* digestion, which indicated that *EcoRI* cuts once at the 3' end of the *cp4 epsps* coding sequence to generate two border fragments. The presence of no more than two border fragments detected in the Southern blot analyses indicates the presence of a single copy of the *cp4 epsps* gene cassette within the insertion site, since multiple copies would result in the detection of more than two unique border fragments. In addition to blots hybridised with the entire plasmid, Southern blot analyses were also conducted with the full-length *cp4 epsps* coding sequence as a probe, cutting with *HindIII* as a restriction enzyme and employing more sensitive methods (Lirette et al., 2000b). The Southern blot analyses were conducted on isolated genomic DNA of soybean 40-3-2, A5403 and A5403 spiked with plasmid PV-GMGT04 DNA. DNA from A5403, showed no hybridisation bands, whereas A5403 DNA spiked with plasmid PV-GMGT04 DNA produced two bands of ~2.5 kb and ~8 kb (as expected from the *HindIII* restriction sites, 155bp and 2705bp, in Figure 2.1.2-1). DNA from soybean 40-3-2 produced a band size of approximately 5.8 kb

(i.e. the primary, functional insert), as well as an additional band of approximately 0.9 kb, which represents a secondary insert that co-segregates with the primary insert (explained below).

Genome walking studies combined with nucleotide sequencing revealed an additional segment adjacent to the 3' end of the NOS 3' polyadenylation signal of the primary, functional insert, which was identified as 250 bp from the *cp4 epsps* element. This sequence corresponded to base pairs 1490-1739 of PV-GMGT04. The stretch of DNA adjacent to the 250 bp *cp4 epsps* segment was also characterised (up to 534 bp) (Lirette et al., 2000b; Goley et al., 2002; Windels et al., 2001) and was shown to be rearranged soybean genomic DNA. The 250 bp *cp4 epsps* segment does not contain a promoter or 3' polyadenylation signal.

Molecular characterisation of the secondary, non-functional insert in soybean 40-3-2

To more accurately define the portion of *cp4 epsps* coding sequence present within the ~ 900 bp *HindIII* restriction fragment, further experiments were conducted. These included cosmid library screening, nucleotide sequencing and PCR analyses (Lirette et al., 2000b). These analyses revealed that the second insert in soybean 40-3-2 consisted of 72 bp (bp 855-926) of the *cp4 epsps* coding sequence located on a 937 bp *HindIII* restriction fragment (Figure 2.2.2.2-1). No other sequences derived from plasmid PV-GMGT04 used in the transformation of soybean 40-3-2 were identified on the 937 bp *HindIII* restriction fragment.

Southern blot analyses to examine the integrity of the *cp4 epsps* gene cassette in the primary, functional insert

In order to assess the intactness of the *cp4 epsps* gene cassette in the primary, functional insert, a series of Southern blots were conducted by the applicant on genomic DNA from soybean 40-3-2 and its control A5403, with element-specific probes.

cp4 epsps coding sequence

Isolated genomic DNA from soybean 40-3-2, A5403 and A5403 DNA spiked with plasmid PV-GMGT04 were digested with *HindIII* (Lirette et al., 2000b). The blot was hybridised with the 32P-labelled full-length *cp4 epsps* coding sequence probe. Soybean 40-3-2 DNA produced a band at approximately 5.8 kb, indicating that the *cp4 epsps* coding sequence is present in soybean 40-3-2. The *cp4 epsps* probe was predicted to hybridise with a 2550 bp *HindIII* band in PV-GMGT04 (2705bp - 155bp = 2550bp, Figure 2.1.2-1), but no fragment of this size was detected in soybean 40-3-2. According to the applicant this indicates that at least one or both of the PV-GMGT04 *HindIII* sites was not transferred to soybean 40-3-2. Subsequent sequence data confirmed the absence of *HindIII* restriction sites in soybean 40-3-2 (Beazley et al., 2001; Lirette et al., 2000b).

E35S promoter

A Southern blot was conducted with soybean 40-3-2 and A5403 DNA cut with *BamHI* and hybridised with the 32P-labeled E35S promoter probe. A single band of 2.9 kb was detected in the soybean 40-3-2 sample, indicating that the E35S element or a portion of it, is present in soybean 40-3-2. Since E35S is located on a 1534 bp *BamHI* fragment of PV-GMGT04 (3158bp - 1624bp = 1534bp, Figure 2.1.2-1) and no fragment of this size was detected in soybean 40-3-2, it was proposed that the *BamHI* site at map number 3158bp in Figure 2.1.2-1 is not present in soybean 40-3-2 (Padgette et al., 1995). Subsequent sequencing of the 5' end of the primary insert confirmed that the enhancer region of the E35S promoter is absent in soybean 40-3-2 (Beazley et al., 2001; Lirette et al., 2000b). The remainder of the E35S promoter is functional, as indicated by the production of the CP4 EPSPS protein and by the tolerance of soybean 40-3-2 to glyphosate.

NOS 3' polyadenylation signal

A Southern blot was conducted with soybean 40-3-2 and A5403 DNA cut with *HindIII* and hybridised with the 32P-labeled NOS 3' polyadenylation signal probe. According to the applicant, at least a portion of the NOS 3' element is present in soybean 40-3-2, since a single band of 5.8 kb was detected in soybean 40-3-2. Two sets of double digestions with *EcoRI* and *BglII* and *EcoRI* and *HindIII* were performed with A5403 and soybean 40-3-2 DNA to assess the intactness of the NOS 3' element. The analyses showed that a 0.8 kb fragment hybridised to the NOS 3' probe in the *HindIII*, *EcoRI* digest where the map-predicted size was 0.3 kb. In addition, a 1.2 kb fragment hybridised to the NOS 3' probe in the *EcoRI*, BglII digest where the predicted size was 0.8 kb. These results indicated that the *HindIII* site at map number 155 and the *BglII* site at map number 10181(Figure 2.1.2-1) are not present in the insert of soybean 40-3-2 (Padgette et al., 1995). Subsequent sequencing of the 3' end of the primary insert confirmed that the NOS 3' element is intact in soybean 40-3-2 (Beazley et al., 2001; Lirette et al., 2000b).

Assessments of other elements from the vector PV-GMGT04 in soybean 40-3-2

Additional Southern blot and PCR analyses were carried out by the applicant to investigate the presence/absence of the other vector elements in soybean 40-3-2. These were: the 35S promoter element P-FMV, the selection marker genes *uidA* (used to identify transformed soybean shoots) and *nptII* (selection of bacteria with plasmids), and the *ori-pUC* sequence used for plasmid replication in *E.coli.* The analyses are described in Padgette et al (1995).

Southern blot with soybean 40-3-2 and control (A5403 DNA) cut with *HindIII* and hybridised with 32P-labeled probes of the P-FMV or the *uidA* coding sequences, showed no presence of either the promoter or selection marker gene in the genome of soybean 40-3-2.

PCR analyses were used to determine the presence or absence of the *ori-pUC* in soybean 40-3-2 and the control A5403. A 5' and a 3' oligonucleotide, identical in sequence to the 5' and 3' ends of the *ori-pUC*, were used in the reactions with genomic DNA from soybean 40-3-2 and A5403. PV-GMGT04 DNA and DNA from a non-commercial glyphosatetolerant soybean transformed with a vector containing *ori-pUC* were used as positive controls. PCR amplification of the DNA from PV-GMGT04 produced the expected size (671 bp). No *ori-pUC* PC-product was found for either soybean 40-3-2 or the control, indicating the absence of an intact *ori-pUC* in soybean 40-3-2.

Likewise, a PCR analysis was used to test for the presence of the *npt//* gene in soybean 40-3-2. Four oligonucleotide primers were used: a 5' and a 3' oligonucleotide for the extreme ends of the *npt//* gene and 5' and 3' oligonucleotide for internal sequences of the gene. The negative controls were A5403 and a non-commercial glyphosate-tolerant soybean negative for *npt//*. The positive controls were PV-GMGT04 DNA and DNA from a non-commercial glyphosate-tolerant soybean positive for *npt//*. The four oligonucleotides were used in combination with each other for a total of four experiments, pairing the 5' and 3' ends, the 5' end and 3' internal, the 3' end and the 5' internal and both internal primers, respectively. Soybean 40-3-2 as well as the negative controls showed none of the predicted *npt//* PCR products in any of the reactions, indicating the absence of an intact *npt//* in soybean 40-3-2.

According to the applicant, subsequent sequence analysis further confirmed the absence of additional elements of plasmid vector PV-GMGT04 in soybean 40-3-2.

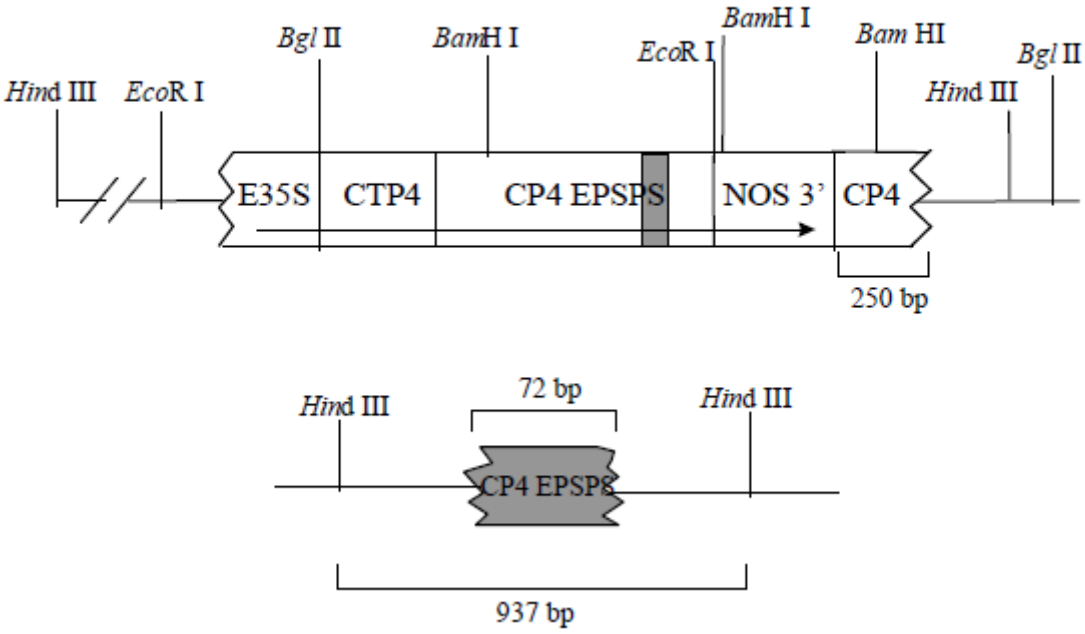

Figure 2.2.2.2-1. Schematic representation of the inserts in soybean 40-3-2 (figure 4 in Technical dossier)

This figure represents the observed DNA inserts in soybean 40-3-2. The primary, functional insert consists of a single cassette containing a portion of the E35S promoter, the chloroplast transit peptide, the *cp4 epsps* coding sequence and the NOS 3' polyadenylation signal. There is an additional 250 bp segment of the *cp4 epsps* coding sequence immediately adjacent to the NOS 3' polyadenylation signal in the primary insert. A second, non-functional insert is present in soybean 40-3-2 located on a 937 bp *HindIII* restriction fragment consisting of 72 bp of the *cp4 epsps* sequence. The shaded region in the *cp4 epsps* coding sequence in the primary, functional insert represents the 72 bp of the *cp4 epsps* coding sequence present in the soybean 40-3-2 secondary insert.

Table 2.2.2.2-1. Restriction analysis of soybean 40-3-2 and PV-GMGT04 (Table 4 in Technical dossier)

Restriction Fragment Size, base pairs [a]					
BamHI		*HindIII*		*EcoRI*	
Predicted Plasmid	Observed 40-3-2	Predicted Plasmid	Observed 40-3-2	Predicted Plasmid	Observed 40-3-2
3166		7959		3202	
	2900		5800		2900
2375		2552		2727	
1536				2503	
1188	1200				1900
1058				1646	
	350		(900) [b]	403	

[a] The values of the plasmid PV-GMGT04 are based on calculated sizes (*see* Figure 1); the values for 40-3-2 are estimated from gel migration relative to molecular weight markers. Bands present in both the experimental and the control lanes are not listed.
[b] Observed with *cp4 epsps* probe.

2.2.2.3 The organisation of the inserted genetic material at the insertion site and methods used for its characterisation

Sequencing of the primary, functional insert and organisation of the elements within the insert in soybean 40-3-2 was assessed by PCR with one primer specific to the genomic DNA flanking the 5' end of the insert (Primer A) paired with a primer located in the genomic DNA flanking the 3' end of the insert (Primer B) (Beazley et al., 2001). The reactions containing soybean 40-3-2 DNA generated a product of a predicted size of ~3.2 kb. This product contained the entire, primary, functional insert in soybean 40-3-2. Control reactions containing no template DNA and soybean A5403 control DNA did not generate a PCR product with the primer set.

The PCR products generated from soybean 40-3-2 were sequenced. The consensus sequence representing the primary, functional insert in soybean 40-3-2 was generated by compiling numerous sequencing reactions conducted on the ~3.2 kb PCR product which spanned the length of the insert. The sequence of the primary, functional insert was compared to the sequence of the plasmid vector PV-GMGT04 and is identical to the portion of plasmid PV-GMGT04 that has been transferred into soybean 40-3-2. As already mentioned, the DNA sequencing of the 3' end of the primary insert revealed the presence of 250 bp of the *cp4 epsps* element adjacent to the 3' end of the NOS 3' polyadenylation signal in the functional insert.

The organisation of the elements within the secondary, non-functional insert in soybean 40-3-2 was assessed by PCR with one primer specific to the genomic DNA flanking the 5' end of the insert (Primer B) paired with a primer located in the genomic DNA flanking the 3' end of the insert (Primer C) (Lirette et al., 2000a). Control reactions containing no template DNA and A5403 control DNA did not generate a PCR product with the primer set. The reactions containing soybean 40-3-2 DNA generated a product of a predicted size of ~1.1 kb. This product contained the secondary, non-functional insert from soybean 40-3-2, consisting of the 72 bp of the *cp4 epsps* coding sequence located on a 937 bp *HindIII* restriction fragment and contained no other DNA derived from plasmid PV-GMGT04 (Figure 2.2.2.2-1). The PCR product was sequenced to define the specific DNA sequence of the 72 bp insert.

Identification of both ends of the inserted DNA and of the genomic flanking sequences

The applicant has performed a PCR-based technique called GenomeWalker to generate PCR products containing DNA at the 5' and 3' ends of the inserted DNA, as well as the DNA flanking the 5' and 3' ends of the primary, functional insert in soybean 40-3-2 (Lirette et al., 2000b). The PCR products were subjected to DNA sequencing, and multiple primers designed to the flanking sequences were paired with insert-specific primers located in i) the E35S promoter (to determine the sequence at the 5' end of the inserted DNA and the 5' flanking genomic sequence), and ii) in the NOS 3' polyadenylation signal (to determine the DNA sequence at the 3' end of the inserted DNA and the sequence of the 3' flanking genomic DNA). PCR products were obtained and sequenced.

The 5' DNA sequence showed that the first 354 bp of the E35S promoter were missing, with the insert beginning at base pair 2347 of PV-GMGT04 (approximate position indicated in Figure 2.1.2-1). This deletion has removed a duplicated portion of the E35S enhancer region. In addition to the 105 bp of E35S promoter which were sequenced, 186 bp of the soybean genomic DNA adjacent to the 5' end of the inserted DNA were identified. The 3' DNA sequence demonstrated that the entire NOS 3' polyadenylation signal (and not only a partial element, as indicated by the Southern blot analyses) was present in soybean 40-3-2 with the inserted DNA ending at base pair 160 of PV-GMGT04 (indicated in Figure 2.1.2-1). The DNA samples from soybean 40-3-2 generated the expected size PCR products of 532 bp for the 5' flanking sequence and 599 bp for the 3' flanking sequence; these are described in Lirette et al (2000b).

2.2.2.4 In the case of deletion(s), size and function of the deleted region(s)

Only a minor part of the 35S promoter is deleted in 40-3-2, and it has not affected the function of the transgene and as such not considered to have any relevant meaning for the risk evaluation.

2.2.3 Information on the expression of the inserted sequences

CP4 EPSPS protein levels were measured by ELISA in soybean 40-3-2 leaf and seed tissues harvested in the 1998 growing season from seven European locations, three sites in France and four in Italy (Hontis., 1998). These field trials were conducted with agronomic practices and field conditions typical of commercial soybean production in the EU and provided environmental situations representative of the European geographical regions where soybean 40-3-2 would be grown. The levels of the CP4 EPSPS protein in leaf and seed samples are summarised in Table 2.2.3-1. In soybean leaf tissue, the mean CP4 EPSPS protein level was 0.502 µg/mg fresh weight (fw). The mean CP4 EPSPS protein level in soybean seed was 0.167 µg/mg fw.

Additional data, generated from samples collected during field trials in USA in the 1992 and 1993 growing seasons, have been reported (Taylor et al., 1999) and are consistent with the EU data. The CP4 EPSPS protein level in soybean leaf tissue from the 1993 field trials ranged from 0.308 - 0.856 µg/mg fw (mean 0.489 µg/mg fw). The CP4 EPSPS protein level in soybean seed from the 1992 trials ranged from 0.258 - 0.378 µg/mg fw (mean 0.301 µg/mg fw), while the CP4 EPSPS protein level in soybean seed from the 1993 trials ranged from 0.166 to 0.287 µg/mg fw (mean 0.218 µg/mg fw).

Table 2.2.3-1. Summary of CP4 EPSPS protein levels measured by ELISA in leaf and seed tissues of soybean 40-3-2 treated and untreated with glyphosate (two applications of glyphosate), from EU field trials (France and Italy) in 1998 (Hontis., 1998). (Adapted from Table 1 in Hontis., 1998).

Soybean Line	Glyphosate - treatment	Tissue	Mean (µg/mg FW)	Range (µg/mg FW)	% CV
40-3-2	**NO**	Leaf	0.524	0.330 - 0.753	26
		Seed	0.172	0.113 - 0.215	18
	YES	Leaf	0.502	0.321 - 0.618	16
		Seed	0.167	0.086 - 0.270	28

Mean: Average protein levels from two soybean varieties derived from 40-3-2, grown at seven sites. Treated plants were sprayed with glyphosate at 0.72 kg active ingredient/hectare at growth stage V3 (~50% of plants have third node visible) followed by a second application three weeks later.

FW = fresh weight
CV = coefficient of variation

2.2.3.1 Part of the plant where the insert is expressed

Production of the CP4 EPSPS protein is expected to occur throughout the whole plant since the E35S promoter has been shown to drive constitutive expression of the encoded protein in genetically modified plants. As seed and leaf are relevant tissues for the safety assessment of soybean 40-3-2, protein levels in these tissues were estimated in the conducted European field trials (Hontis., 1998).

2.2.3.2 Expression of potential fusion proteins and analyses of open reading frames

To assess the presence of proximal genetic regulatory elements, such as transcriptional promoters and polyadenylation signals, the DNA sequences flanking the 72 bp secondary, non-functional insert and the 250 bp *cp4 epsps* segment located adjacent to the 3' end of the NOS 3' polyadenylation signal of the primary functional insert, were compared to known plant promoter and polyadenylation signals available in public domain databases. In addition, bioinformatics analyses were conducted to determine the potential for toxicity, allergenicity or pharmacological activity of putative polypeptides encoded by the 5' and 3' junctions of the inserted DNA sequences (Silvanovich et al., 2000).

For the assessment of potential genetic regulatory elements, a 100% sequence identity was considered biologically relevant. With the DNA sequences containing and flanking the 72 bp and 250 bp *cp4 epsps* DNA segments as query sequences, three promoter databases were separately searched for similarity towards known genetic promoter elements. No promoter elements were identified to any of the 860 bp of DNA containing the 250 bp DNA segment of *cp4 epsps* in the databases. The 1103 bp segment of DNA that contained the 72 bp DNA segment of *cp4 epsps* and its associated flanking sequences yielded two hits which were determined by the applicant to be contextually inconsistent relative to any identified open reading frames. Possible polyadenylation signals were also analysed. Only the most dominant sequence elements were considered in this analysis, indicating that a complete set of polyadenylation signals was not observed in either reading frame of the 72 bp *cp4 epsps* segment and its associated flanking DNA or the positive DNA strand of the 250 bp DNA segment of *cp4 epsps* and the DNA which flanks this sequence.

To assess the potential similarity towards toxins, allergens or other pharmacologically active proteins, putative polypeptides derived from the DNA sequences containing the 72 and 250 bp *cp4 epsps* segments were translated and evaluated with bioinformatics tools. All six possible reading frames originating or terminating within the 72 bp segment and extending

into or from genomic sequences and the three forward reading frames originating within the 250 bp segment and terminating in the 3' flanking genomic sequence have been translated. To maximise the number of putative polypeptides, a stop codon rather than a start codon (ATG) was used to define both the N-terminus and the C-terminus of each putative polypeptide. Translation was therefore conducted from stop-to-stop codon. The amino acid sequences of the putative polypeptides spanning the *cp4 epsps* coding sequence and the corresponding genomic flanking sequences were compared with the sequences contained in the ALLPEPTIDE, TOXIN4 and UPDATE2 databases with the FASTA sequence alignment tool to assess structural similarity. Each putative polypeptide was also screened against the allergen database UPDATE2 with an algorithm that scanned for a window of eight linearly contiguous amino acids. According to the applicant the results of these bioinformatics searches revealed no biologically relevant similarities between the putative polypeptides encoded by the DNA sequences containing and flanking the 72 and 250 bp *cp4 epsps* segments and toxins, allergens or other proteins available in public databases at the time.

2.2.3.3 Further characterisation of the 3' flanking sequence of the primary, functional insert and tests for the presence/absence of fusion proteins

The DNA sequence adjacent to the 3' end of the primary, functional insert is described in Lirette et al (2000b) and Goley et al (2002). Further characterisation of this sequence is described in the Monsanto technical report by Mittanck et al (2002). The report assesses if RNA transcript containing sequences adjacent to the 3' end of the functional insert could be identified in soybean 40-3-2. Northern blot analyses in this study indicated that secondary RNA transcripts, including a ~7.4 kb transcript (the primary CP4 EPSPS transcript is ~1.5 kb as described in Lirette et al (2000b)), are produced at very low levels and contain the DNA sequence flanking the 3' end of the primary, functional insert. Further analyses by strand-specific northern blotting revealed that these secondary transcripts are transcribed in the same orientation as the *cp4 epsps* transcript produced by the primary, functional insert. Quantification experiments showed that the abundance of the ~7.4 kb secondary transcript was approximately 75 times lower than the ~1.5 kb *cp4 epsps* transcript produced by the primary, functional insert, while other transcripts were produced at even lower levels. The results indicate that these secondary transcripts were products of incomplete transcriptional termination by the NOS 3' polyadenylation sequence. Subsequent RT-PCR analyses confirmed that the secondary transcripts initiated within the functional insert from the E35S promoter and continued through the NOS 3' polyadenylation sequence into the soybean genomic DNA flanking the 3' end of the functional insert. According to the applicant, the potential of the secondary transcripts to produce a protein product was estimated as highly unlikely based on protein analyses. Furthermore, according to the applicant the analyses also showed that the only CP4 EPSPS protein found in soybean 40-3-2 was the well characterised 46 kDa full-length CP4 EPSPS protein (Rogan et al., 1999), and that no immunoreactive proteins of molecular weights greater than 46 kDa were detected in extracts of soybean 40-3-2.

Bioinformatics analyses on the open reading frames (ORFs) present in the region beyond the 3' end of the *cp4 epsps* coding region, including the region of soybean genomic DNA flanking the 3' end of the functional insert, did not show any homology to known toxins or allergens.

Overall the data analyses provided by the applicant indicate that even though soybean 40-3-2 also produces secondary RNA transcripts that encompass soybean genomic sequences flanking the 3' end of the transgenic insert, only the full-length CP4 EPSPS protein is expected to be produced.

2.2.4 Updated bioinformatics analyses provided by the applicant

The EFSA GMO Panel requested in 2008 and 2010 updated bioinformatics analyses on soybean 40-3-2 in connection with the renewed application EFSA-GMO-RX-40-3-2. The applicant accommodated EFSAs request(s). The most recent updates are described in Monsanto reports by Tu and Silvanovich (2010a,b).

The databases used for the BLASTn analyses were the GenBank EST database (EST_2010) and the GenBank non-redundant nucleotide database (NT_2010). The database used for the BLASTx analysis was the GenBank non redundant amino acid database (NR_2010). The allergen, gliadin, and glutenin sequence database (AD_2010, release 175.0) was obtained from FARRP (2010), downloaded from NCBI and formatted for use in these bioinformatic analyses. It is referred to as the PRT_2010 database. The toxin database is a subset of sequences derived from the PRT_2010 database that was selected with a keyword search and filtered to remove likely non-toxin proteins. It is referred to as the TOX_2010 database. Complete descriptions of the databases are found in Tu and Silvanovich (2010a).

According to the applicant, the results from the updated bioinformatics analyses indicate that in the unlikely event that any of the ORFs spanning the junctions of the insert were to be transcribed and translated, the translation products would not share significant similarity to known allergens, toxins, or other bioactive peptides.

According to EFSA the updated analyses do not warrant changes to the prior risk assessments of soybean 40-3-2 (EFSA 2010b). Tables AI-1, AI-2 and AI-3 (Appendix I) give a summary of the bioinformatics analyses that were performed. All analyses are included in Tu and Silvanovich (2010b).

2.2.5 Genetic stability of the insert and phenotypic stability of the GM plant

2.2.5.1 Genetic stability of the insert in soybean 40-3-2

The applicant has performed Southern blot analyses with DNA extracted from leaf tissues originating from soybean 40-3-2 generations R3 and R6 seeds (Kolacz & Padgette., 1994). Control DNA was extracted from leaf tissues of soybean A5403, which has the same genetic background as soybean 40-3-2. The DNA samples were digested with *HindIII*, which cleaves in the soybean DNA flanking the *cp4 epsps* insert and releases a 5.8 kb fragment, as previously described (shown in Table 2.2.2.2-1).

The blots were hybridised with the 32P-labelled PV-GMGT04 probe. The results show that identical 5.8 kb bands were found for the soybean 40-3-2 R3 and R6 generations, which indicates that the *cp4 epsps* insert is stable over at least four generations.

2.2.5.2 Phenotypic stability of the glyphosate tolerant trait in soybean 40-3-2

The applicant has performed several crosses with conventional breeding techniques with soybean 40-3-2. Trait characteristics in the progenies from these crosses were monitored phenotypically at the whole plant level by application of glyphosate herbicide. In one case, soybean 40-3-2 was crossed with several traditional soybean varieties. According to the applicant, the progeny of these crosses consistently segregated three tolerant to one sensitive plant, as expected for a homozygous trait. The results of the segregation data, including the Chi-square (X^2) analysis, are illustrated in Table AI-4 (Appendix I). None of the X^2 values indicate a significant difference between observed and expected segregation ratios for soybean 40-3-2. The results of the X^2 analysis indicate that a single functional DNA insert is integrated in the plant nuclear genome of soybean 40-3-2 and that it is stably inherited as a single locus, following a Mendelian one-locus model. In a second case, two families of commercial breeding soybean lines were assessed for their inheritance patterns. According to the applicant, the first commercial breeding line (DBL3201AOX) was at least 24 generations from the initial soybean 40-3-2 transformant, and the second breeding line (DKB2301A1R) at least 29 generations. Both commercial breeding lines were generated by the traditional breeding practice of backcrossing soybean 40-3-2 with an elite traditional variety. The subsequent F1 progeny were tested by a PCR-based assay to determine zygosity. The F1 progeny were determined to be heterozygous and were then selfed. The resulting F2 progeny were assessed by a PCR-based zygosity assay to determine the segregation pattern. Based on an expected 1:2:1 segregation pattern, the experiment with these two breeding lines (DBL3201AOX and DKB2301A1) were predicted to yield 7.5 null to 15 heterozygous to 7.5 homozygous segregants for the 29 and 30 plant populations, respectively. The segregation patterns are presented in Table AI-5 (Appendix I), and show

that soybean 40-3-2 segregated according to a Mendelian one-locus model during the breeding of the commercial lines.

Published studies have also evaluated the agronomic and phenotypic characteristics of several generations of soybean 40-3-2 (Delannay et al., 1995) and the impact of different glyphosate treatments on gametic selection and segregation patterns in soybean plants hemizygous for *cp4 epsps* (Walker et al., 2005). Overall, the studies by the applicant and the studies by Delannay et al (1995), and Walker et al (2005), show that the glyphosate-tolerance trait in soybean 40-3-2 has been consistently inherited in a Mendelian fashion over multiple generations and across diverse germplasms.

2.3 Conclusion

The applicant has provided sufficient analyses to characterise the DNA inserts, number of inserts, integration sites and flanking sequences in the soybean genome. The results show that one functional copy of the *cp4 epsps* gene only, is present in the soybean 40-3-2 genome. No other functional vector genes were found. Updated similarity searches in 2010, with databases of known toxins and allergens did not indicate a potential production of harmful proteins or polypeptides caused by the genetic modification. Southern blot analyses and segregation studies show that the introduced gene is stably inherited and expressed over multiple generations along with the phenotypic characteristics of soybean 40-3-2. The VKM GMO Panel concludes that the molecular characterisation of soybean 40-3-2 does not indicate a safety concern.

3 Comparative assessment

The application on GM soybean 40-3-2 for renewal of the authorisation of existing products (EFSA-GMO-RX-40-3-2 (8(1) (a) and 20(1)(a)) refers to the original field trials performed in Puerto Rico (1991 and 1992) and in the USA (1992-1993). These were subsequently extended with compositional data collected from field trials in France (1998) and Italy (1998) (Taylor 2005, in the application for cultivation, EFSA/GMO/NL/2005/24). The design of the field trials varied considerably, and was not in accordance with the current EFSA guidelines (EFSA 2006). The data from all these field trials were previously evaluated by VKM in 2007

The renewal application provides new compositional data from a field study in Romania in 2005 (Harrigan et al., 2007). This study was designed accordingly to the EFSA document, the soybean 40-3-2 was compared to a conventional counterpart (Dekabig) and to a set of conventional varieties with comparable genetic background. Finally, the technical dossier refers in the renewal application to a broad range of field studies performed over several years with multiple backgrounds of 40-3-2 compared to different varieties of conventional controls (McCann et. al., 2005).

3.1 Production of material for comparative assessment

A comparative approach was used to evaluate the food safety of soybean 40-3-2 and products derived from it to determine whether this GM soy is as nutritious and safe, as conventional soybeans and derived products. Soybean 40-3-2 is intended to be used in the same way as any commercial soybean variety in production and manufacturing of food and feed products thereof.

The genetically modified soybean 40-3-2 was compared to the non-transgenic Asgrow variety A5403, in most compositional studies. Asgrow A5403 is the commercial soybean variety originally used to produce the transformed event 40-3-2. When the GM event 40-3-2 was bred into another genetic background, the corresponding non-GM variety was used as conventional counterpart (Dekabig).

The field trials were performed at five sites in Romania in 2005 (Harrigan et. al., 2005). They were replicated and included soybean 40-3-2, and the conventional counterpart (Dekabig) along with three to four commercially available soybean varieties. The soybeans were planted in a randomised complete block design composed of three blocks or replications. The Roundup Ready soybean 40-3-2 plots were treated with three applications of Glyphosate (Roundup Ultra herbicide): at pre-emergence, at early post-emergence (V4-V6 stage), and at late post-emergence (V8 or 30 inches tall, whichever came first).

The study performed by McCann et al., 2005, included three growth seasons; 2000, 2001 and 2002 in the United States and Canada. The trials in 2000 and 2001 used a broad range of totally 25 different varieties of GM soybeans resistant to glyphosate and 25 different varieties of conventional controls, while in 2002 a total of 16 different GM soybeans were compared to 16 conventional controls. The genetically modified varieties were treated in-season with agricultural herbicide (Roundup) applications according to the labelled recommendations.

Statistical analysis

In the European field trial in 1998 (Taylor et al., 2005), the test and conventional varieties were divided into five groups, and differences among the groups were tested. The two varieties of Roundup Ready soybean (AG2101 and AG2401) untreated and treated with glyphosate made a total of four groups, and these were compared to group five containing the conventional varieties. The statistical analysis was performed with a mixed linear model (PROC MIXED) in the SAS statistical analysis program (SAS Institute, Inc. 1990).

The Romanian study was also analysed with a mixed-model analysis of variance. For each compositional component the forage and harvested seeds from soybean 40-3-2 was compared to the conventional control. From the reference substances, a population tolerance interval was determined. A tolerance interval is an interval that with a specified degree of confidence contains at least a specified proportion, p, of an entire sampled population for the parameter measured. For each analyte the 99% tolerance interval is expected to contain, 99% of the quantities expressed in the population of conventional references, with 95% confidence. Each tolerance interval estimate was based upon one summary value for each unique reference substance.

3.2 Compositional analysis

3.2.1 Romanian field trial (2005)

For the food fraction, soybean seeds were harvested and analysed for nutrient composition, as proximates (the macronutrients protein, fat, ash, moisture and carbohydrate by calculation), fiber fractions, amino acids, fatty acids, anti-nutrients (phytic acid, trypsin inhibitor, lectin, stachyose and raffinose) and isoflavones (daidzein, genistein and glycitein. In forage only proximates and fiber fractions were analysed. In total 63 compounds were analysed, 56 in seeds and seven in forage. This is in accordance with the recommendations by OECD (2001).

Proximate and fiber composition of forage and harvested seeds

Data for the content of proximates and fiber in forage are presented in Table AI-6 and for seeds in Table AI-7 (Appendix I). These results demonstrate comparable levels of proximate compounds (macronutrients) and fiber in the harvested seeds and forage of soybean 40-3-2 and the conventional control. The amount of acid detergent fiber (ADF) in forage from 40-3-2 was 31.93% DM (dry weight of sample) and 30.26% DM in the control, a small but statistically significant increase in the GM compared to the control ($p<0.05$). The values for all compounds analysed in forage and grain were within the 99% tolerance interval for commercial varieties, grown in the same field trial.

Amino acid composition

The content of the 18 amino acids measured in harvested seed from soybean 40-3-2 was comparable to that of the conventional control (Dekabig) (Table AI-8, Appendix I). In the combined site analysis a statistically significant difference was observed for isoleucine and valine, with reduced amounts in the GM soya compared to the control. The differences were however small (around 5%), and the values were within the 99% tolerance interval for the commercial varieties grown in the same field trial.

Fatty acid composition

The content of fatty acids in seeds from soybean 40-3-2 was comparable with the levels observed in the conventional control (Dekabig), and there were no statistically significant differences in the combined site analysis (Table AI-9, Appendix I).

Isoflavones

The levels of isoflavones (daidzein, genistein, glycitein) of harvested seed from soybean 40-3-2 are presented in Table AI-10 (Appendix I). A statistically significant difference was observed for the level of genistein, which showed a slightly higher level in the conventional control ($p<0.05$). The individual values were within the 99% tolerance interval for commercial varieties, and within the range of the reported historical controls. This indicates that the levels of isoflavones in 40-3-2 were within the same population range as those of conventional, commercially grown soybean.

Antinutrients

The contents of stachyose, raffinose, phytic acid, trypsin inhibitor, and lectin in seed from 40-3-2 were comparable with the level observed in the harvested seed of the conventional control (Table AI-11, Appendix I). No statistical significant differences were observed in the combined site analysis of these five analysed antinutrients.

To summarise the compositional data from the Romanian study, across sites, the evaluation revealed that four of the 49 comparisons were statistically significantly different ($p<0.05$) between the 40-3-2 and the conventional counterpart. These four were ADF in forage, and isoleucine, valine and genistein in seeds. When these differences were evaluated per site the levels were different at only one of the five trial sites. The differences were small and fell within the normal variation found in the reference soybeans included in the study and also of those described in the ILSI (2006) and the USDA-ISO (2006) databases. Also for other constituents, statistically significant differences were found for 20 compounds at one of the five sites. For four other compounds, differences were seen at two of the five sites. In all cases the differences were small, and the levels fell within the normal variation of the reference lines and within the range of reported historical controls.

3.2.2 Field trials from the United States and Canada (2000, 2001 and 2002)

Additional compositional comparisons of the content of proximates, lectin, trypsin inhibitor, and isoflavones in seeds of soybean 40-3-2 crossed into diverse genetic backgrounds with the composition of corresponding conventional non GM counterparts grown together with the 40-3-2 in the field experiment (see 3.1) were performed by McCann et al. (2005). The field trials were conducted in 2000, 2001 and 2002 in the United States and Canada and the GM crop was treated with Roundup. The study was designed to study if the composition of glyphosate tolerant soybeans remains substantially equivalent to conventional soybeans over the course of several years and when introduced into multiple genetic backgrounds. Typical for the measured levels of all nutrients and antinutrients are that they vary depending on the environmental conditions, the cultivar grown, and the method used. The results of the study showed no differences between 40-3-2 and the conventional soybean varieties concerning mean and range of nutrients. The nutrient levels are also similar to the levels reported in the ILSI Crop Composition Database.

Other compositional trials

Several other studies have reported on the compositional equivalence of soybean 40-3-2 and commercial soybean varieties. One study investigating the content of proximates, isoflavones, monosaccharides, trypsin inhibitors and phytate in full fat soybean meal concluded that the overall chemical composition was similar and within normal ranges given for other soybean products (Hemre et al., 2005). Novak and Haslberger (2000) reported substantial equivalence of soybeans 40-3-2 to commercial varieties with respect to protease-inhibitors; lectins, isoflavones and phytate.

Since the first safety assessment of soybean 40-3-2 (VKM 2007), there has been some studies linking glyphosate treated plants to changes in nutrient composition (Zobiole et al., 2010, Bøhn et al., 2014). These studies report statistically significant differences in protein

content, total amino acids, some fatty acids and/or zinc content in the glyphosate-treated compared to untreated soybeans. Other studies have indicated that applications of glyphosate have no effects on e.g isoflavone levels in glyphosate-resistant soybeans (Duke et al 2003). Henry et al. (2011) studied the effect of glyphosate on the concentration of macronutrient and micronutrients in soybean 40-3-2 and RR2Y soybean (Roundup Ready 2 Xtend soybeans, second generation soybean with several copies of the *epsps* gene). Although there are differences in accumulation of macro and micronutrients in the two cultivars tested, compared to the control, no consistent effect due to glyphosate treatment was found. If soil nutrient levels are properly maintained in fields, glyphosate is unlikely to cause macro and micronutrient deficiencies in soybean fields (Henry et al., 2011). In a recent work by Duke et al. (2012b) available studies (232 references) on glyphosate effects on plant mineral nutrition were reviewed. Their main conclusion is that the literature on the effects on mineral nutrition in glyphosate tolerant GMO (GT-GMO) crops is conflicting. Most of the literature indicates that the mineral nutrition is not affected by either the GT-GMO trait or glyphosate application and that the yield data on GT-GMO crops do not support that there are mineral or disease problems specific to GT-GMO crops.

3.3 Agronomic traits and GM phenotype

Comparative assessments of the phenotypic and agronomic characteristics of soybean 40-3-2 and its conventional counterpart have been conducted, based on field trials in the EU at locations representative of the soybean cultivated area across several years (Italy 1994, 1996 and 1997; France 1994). Characteristics evaluated included date of emergence, % of emergence, plant count, plant height, vigour and colour, morphological changes, date at 50% flowering, the difference in susceptibility to insects, nodes per plant, pods per plant, % lodging, % leaf drop, yield and % moisture. On the basis of the documents presented by the applicant, the EFSA GMO Panel declared that soybean 40-3-2 is substantially equivalent to traditional soybeans and that "*no meaningful difference between soybean 40-3-2 and its conventional counterpart were identified, except for the introduced glyphosate tolerance trait.*"

The agronomic and phenotypic equivalence of soybean 40-3-2 compared to conventional soybean has also been assessed in field tests conducted in the USA and Puerto Rico (1991-1994), in Argentina (1993-1994) and in Canada (1993 and 1994).These studies were described in the notification C/UK/94/M3/1 submitted under Directive 90/220/EEC. The results of these field tests established that there were no differences in survivability, no change in yield and that the introduced trait has no influence on dissemination when comparing soybean 40-3-2 to the near-isogenic control (A5403), which had the same genetic background as 40-3-2.

The glyphosate tolerance trait expressed in 40-3-2 has been transferred into more than one thousand commercial soybean varieties by traditional breeding techniques (CERA, 2014).

Soybean 40-3-2 was first planted commercially in the USA in 1996. Since then, 40-3-2 has become the GM crop with the largest hectares planted in the world. According to CERA (2014), no significant differences in morphology, seed production (yield), agronomic characteristics (such as time to flowering and pod set, or vigor) and tendency to weediness have been reported.

Prior to and after the commercial introduction of soybean 40-3-2 in North America, various research groups have published data on yield, plant height and glyphosate tolerance (Delannay et al., 1995; Elmore et., 2001,a,b), the susceptibility of 40-3-2 to insect pests (McPherson et al., 2003; Morjan & Pedigo, 2002), nematode damage (Koennig, 2002; Yang et al., 2002) and diseases, including fungal pathogens (Harikrishnan & Yang, 2002; Lee et al., 2000; Mueller et al., 2003; Njiti et al., 2003; Sanogo et al., 2001; Sanogo et al., 2000). These investigations have largely confirmed initial field observations that 40-3-2 is equivalent to traditional soybean in terms of growth habit, yield potential and disease and pest resistance and thus pose no increased weediness or pest potential. Elmore et al. (2001b) reported 5 % yield suppression in soybean 40-3-2 treated with glyphosate. The slightly reduced yield appeared to be associated with the introduced gene or its insertion process rather than glyphosate itself.

3.4 Conclusion

The VKM GMO Panel has considered the available literature on compositional data and found no biologically meaningful differences between soybean 40-3-2 and the conventional non-GM control, except small intermittent variations. The data presented do not show unintended effects as a result of the genetic modification. The VKM GMO Panel concluded that soybean 40-3-2 is compositional, agronomical and phenotypically equivalent to its conventional counterpart, and other conventional soybean varieties, except for the introduced glyphosate tolerance trait.

4 Food and feed safety assessment

4.1 Previous evaluation by the VKM GMO Panel

In an earlier risk assessment of soybean 40-3-2 the VKM GMO Panel concluded that the soybean 40-3-2 is nutritionally equivalent to conventional soybean varieties (VKM 2007). This was based on data from feeding studies with processed and unprocessed soybean on rats, broilers, pigs, heifer and channel catfish (*Ictalurus punctatus*).

The VKM GMO panel has now evaluated a number of feeding studies of various length performed in rodents given processed and unprocessed soybean 40-3-2 in the diet. These studies indicated no toxicity related to the genetic modification. It is unlikely that the inserted gene will introduce a toxic or allergenic effect in food or feed based on soybean 40-3-2 compared to conventional soybean.

4.2 Product description and intended uses

Soybean 40-3-2 is not only one of the first GMs cultivated, but it is still the dominated soybean variety grown worldwide. It was first cultivated in USA and Argentina in 1996, and subsequently commercialised in Canada, Uruguay, South Africa, Brazil, Romania and Paraguay. The production in Romania between 1999 and 2006 was prior to the accession to the EU in 2007. Currently the amounts produced exceed 90% of total soybean production area in the USA and Argentina. At discontinuation of soybean 40-3-2 production in Romania in 2006 it was cultivated on 84% of the land devoted to soybean cultivation.

The genetic modification in soybean will not impact the existing post-harvest production processes used for soybeans. The major soybean commodity products are seeds, oil, meal and protein concentrates/isolates. Conventional soybean concentrates are a common feed ingredient in Norwegian salmon feed (www.mattilsynet.no).

Unprocessed soybeans are not suitable for food and their use in animal feed remains limited because they contain anti-nutrients such as saponins, trypsin inhibitors and lectins (OECD 2012). However, aspirated grain fractions, forage, hay, hulls, seeds, and silage are also used as feed to a limited extent, primarily to cattle (OECD 2012). Adequate heat processing inactivates most of the biological activity of these factors. Whole soybeans are utilised to produce food products such as soy sprouts, baked soybeans, roasted soybeans, full fat soy flour and the traditional soy foods (miso, soy milk, soy sauce, and tofu) (OECD 2012). The processing steps used in food manufacturing of soybean are shown in Figure 4.2-1 adapted from the Technical dossier. The first step in processing most soybeans is to separate the oil, either by solvent extraction or by expelling.

All GM soybean products are produced and processed for use in food, animal feed and industrial products in the same way as other commercial soybean and according to the applicant the commercial experience since 1996 has confirmed that this has been the case. The major soybean commodity products are seeds, oil, and meal.

The soybean 40-3-2 and all food, feed and processed products derived thereof are expected to replace a portion of similar products from commercial soybean, with total consumption of soybean products remaining unchanged.

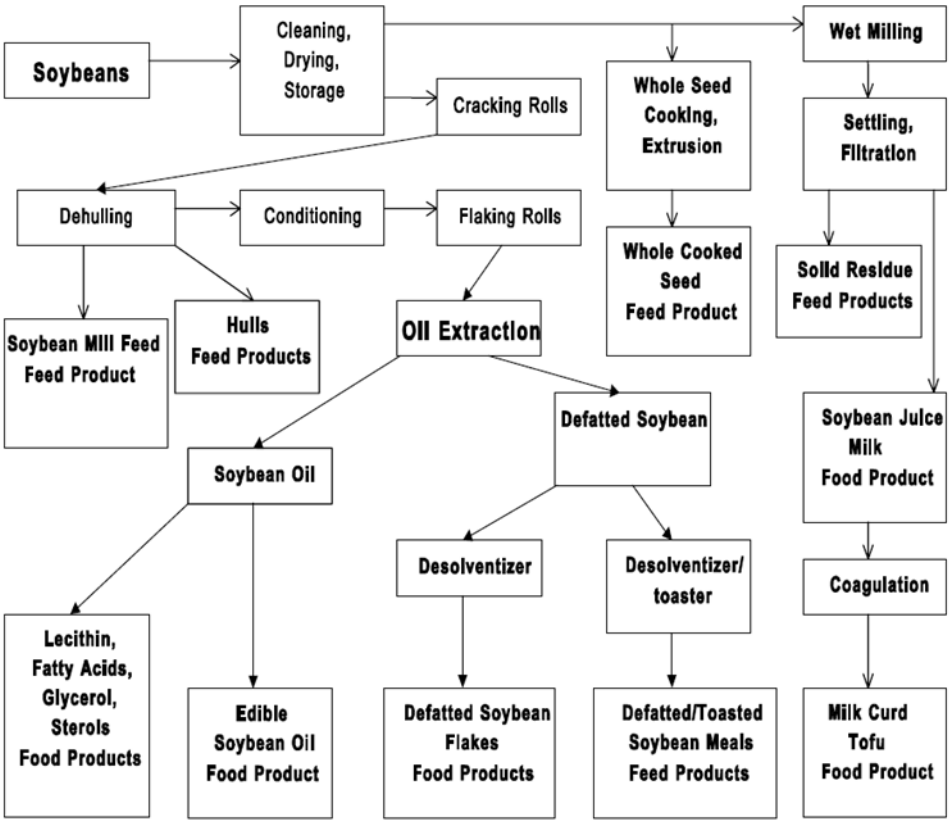

Figure 4.2-1. Processing of soybean, adapted from Waggle and Kolar, 1997, Technical dossier

4.3 Effects of processing

The processing steps which are used to produce the various soy products are shown in figure 4.2-1, above. Soybeans are first cracked and de-hulled, then heated to approximately 60 degrees, ground to flakes with rollers, and are then treated with solvent to remove the oil. The flakes are toasted, cooled and ground. During these processes, proteins in soy are subjected to harsh conditions, such as thermal processing, changes in pH, reducing agents, mechanical shearing, and so on, which will lead to denaturation and loss of protein function. Intermediate temperatures (55°C) will reduce the activity of the CP4 EPSPS enzyme, while it

will be completely inactivated at higher temperatures (65° and 75°C). pH values < 4, will also reduce enzymatic activity (Effective range of enzyme is pH 4-11). The CP4 EPSPS enzyme is degraded in foods like tofu and soybean paste (Kim et al. 2006b). Studies by Wu et al. (2012 a, b) and Tian et al (2014) showed that the CP4 EPSPS protein and *cp4 epsps* gene in soybean 40-3-2 was degraded when preparing foods such as tofu, soybean paste and soybean meal. The heat treatments used by Wu et al. (2012) were boiling, autoclaving or heating by microwave oven. Autoclaving, when used to manufacture soy drink, textured vegetable protein, soybean meal, etc. generated more degradation of CP4 EPSPS-protein then boiling and microwave treatment. The processing methods used by Tian et al. (2014) were dry heat treatment, wet heat treatments and extrusion. They used different temperatures (e.g 75°C to 135 °C) and different times (3 to 30 minutes). Degradation of the *cp4 epsps* gene and CP4 EPSPS protein depended on time and temperature. As temperature rose from 90°C to 150°C the CP4 EPSPS protein content was reduced from 4,19 % to 0.54 %, and was not detectable at 165°C. The 483-bp *cp4 epsps* gene was not detected after dry heating, wet heating, or extrusion at 120 °C with a 39 % moisture content (Tian et al. 2014).

Similar results were found by Fernandes et al. (2013) when baking the maize bread broa (a Portuguese oven baked bread made with polenta) containing 11 % of TC1500 and 20 % MON810 maize flour. Under these conditions the majority of DNA and proteins in maize was denatured, which also applies to the CP4 EPSPS protein and *cp4 epsps* gene in processed maize products (Dien et al., 2002, Hammond & Jez 2011, Fernandes et al., 2013).

Another study quantified the levels of CP4 EPSPS proteins in full fat soybean meal (FFSBM) and in extruded salmon feeds with soybeans, only trace levels of CP4 EPSPS was found in the FFSBM product, and the levels in the fish feed was reported to be non-detectable (<0.1%) (Sanden et al., 2005).

4.4 Toxicological assessment of soybean 40-3-2

The toxicological assessment is based on results available from testing in mice, rats, broiler chickens, pigs, dairy cows, salmon, catfish and rabbits.

Animal studies have utilised various formulations of soybean 40-3-2 such as purified CP4 EPSPS protein, protein concentrate from soybean 40-3-2 or whole GM food and feed. Protein concentrate is about 70% soy protein and is basically defatted soybean flour without the water-soluble carbohydrates. It is widely used as a functional or nutritional ingredient in a wide variety of food products, mainly in baked foods, breakfast cereals, some meat products and as fish feed for aquaculture.

A safety testing programme has been conducted on soybean 40-3-2 within the Russian Federation and summarised in Tutelyan (2013).The research and testing is claimed compliant with national requirements (MY 2.3.2.2306-07 “Medico-biological safety

assessment of genetically-engineered and modified organisms of plant origin"). The content of these requirements and the exact design of the respective studies have been difficult to assess for the VKM GMO panel. Still, the testing conducted in the Russian Federation is deemed valuable for the risk assessment of soybean 40-3-2. This is due to the programme being rather extensive with several studies conducted and many parameters monitored. Also, the studies are of particular interest since these are the only studies conducted with a soy protein concentrate, a main ingredient when blending fish feed. A brief summary is presented in Appendix III.

4.4.1 CP4 EPSPS protein used for safety assessment

Submitted data demonstrated a low expression of the CP4 EPSPS protein in soybean 40-3-2 (<0.1%). The protein was not detectable in soybean oil and showed no meaningful amino acid sequence homology to known toxic proteins (UK-ACNFP, 1995). Also in vitro digestion studies with simulated gastric fluid, demonstrated that CP4 EPSPS is rapidly degraded at conditions mimicking the stomach (Harrison et al., 1996). Rapid digestion of microbially produced CP4 EPSPS, as well as of CP4 EPSPS extracted from soybean 40-3-2, has later been confirmed in studies with pepsin and pancreatin digestion assays (EFSA 2010; Okunuki et al., 2002; Chang et al., 2003; Kim et al., 2006b).

There are also studies showing that the CP4 EPSPS protein is easily degraded in eggs, liver and faeces(Ash et al., 2003) as well as in muscle tissue (Jennings et al., 2003; Zhu et al., 2004) of hens, pigs and rats fed soybean 40-3-2. Thus, digestion seems to result in levels where no detectable protein is absorbed in the investigated tissues. Taken together, the digestive fate of the CP4 EPSPS protein and the *cp4 epsps* gene, indicate that no CP4 EPSPS protein accumulate in tissues of tested organisms, and that only small fragments of DNA can be detected. Comparative studies on the digestive fate of endogenous and transgenic plant gene sequences, show that these genes behave in a similar way.

4.4.1.1 Acute toxicity testing

An acute mouse oral gavage study with the CP4 EPSPS protein to assess any potential toxicity associated with the CP4 EPSPS protein has been performed by Harrison et al. (1996). The study was compliant with the US EPA FIFRA GLP (40 CFR Part 160), EU-directive 88/320/EC) and acute oral toxicity guidelines of U.S. EPA and OECD (U.S. EPA Health Effects Test Guidelines. OPPTS 870.1100; Acute Oral Toxicity (US EPA 2002), OECD Guideline for Testing of Chemicals; Method No. 420: Acute Oral Toxicity-Fixed Dose Method; July 17, 1992 (OECD 2001)). The CP4 EPSPS protein was over-produced and purified in *E. coli* and administered to mice at a single high dose. Three groups of mice (each 10/sex) were dosed with 49, 154 and 572 mg/kg body weight (bw) respectively. These doses correspond to 40, 100 and 400 mg/kg bw CP4 EPSPS protein based on the level of purity of the protein and ELISA analyses of the dosing solutions. A control group received bovine serum albumin

(BSA) at a target dosage of 400 mg/kg bw (actual dose was 363 mg/kg bw) in the same solution and delivery volume as the test substance. The second control group was administered the carrier solution (vehicle control) only; 50 mM sodium bicarbonate. No treatment-related adverse effects were observed in mice administered the CP4 EPSPS protein by oral gavage at the highest dose tested, i.e. 572 mg/kg bw. There were no statistically significant ($p \leq 0.05$) differences in body weight, cumulative body weight, or food consumption between the vehicle and bovine serum albumin protein control groups and CP4 EPSPS protein treated groups. The study concluded that the acute oral LD50 of microbiologically derived CP4 EPSPS protein in the mouse is higher than 572 mg/kg bw. A similar study with a similar conclusion has been published by Brooks in 2000 (Brooks et al 2000).

The CP4 EPSPS protein has previously been assessed by VKM in other genetically modified glyphosate tolerant crop varieties including soybean (MON 89788, MON87705, MON 87701 x MON 89788, MON 87769 x MON 89788 and Soybean 305423 x 40-3-2), cotton, rape seed and several lines of maize (see food and feed risk assessments of several maize e.g. NK603, NK603 x MON810; 1507 x NK603; MON 863 x NK603; MON 1445 x MON 531, GA21; and more).

The VKM GMO Panel agrees with EFSA in the opinion that acute toxicity testing of the newly expressed proteins is of little additional value to the risk assessment of the regular human and animal consumption of food and feed derived from GM plants and is therefore not taken into account in this risk assessment (EFSA, 2011a).

4.4.1.2 Toxicological assessment of new constituents other than proteins

No new constituent other than the CP4 EPSPS protein is expressed in soybean 40-3-2 and no relevant changes in the composition of soybean soybean 40-3-2 were detected by the compositional analysis.

4.4.2 Toxicological assessment of the whole GM food / feed

The potential toxicity of soybean 40-3-2 expressing the *cp4 epsps* gene has been assessed in repeated dose toxicity studies in rodents.

Although the comparative analyses provided showed soybean 40-3-2 to be compositionally equivalent to conventional soybean varieties (except for the CP4 EPSPS protein), the applicant submitted four rat feeding studies with the GM soybean (two 4week toxicity studies, one 15week study on immunotoxicity and one 13week subchronic toxicity study).

According to the updated version of the EFSA guidance for risk assessment of food and feed from genetically modified plants (EFSA, 2011a), the experimental design should always include the following test materials: the GM plant exposed to the intended herbicide, and the

GM plant treated with the conventional herbicide management regimes. Some of the studies provided by the applicant are not in accordance with the suggested experimental design in the last EFSA guidance document on risk assessment (EFSA, 2011a). The Norwegian GMO Panel agrees on the importance of including GM plants treated with the intended herbicide in comparative analysis (composition, agronomic traits, food and feed safety assessments), but recognises that the applicant submitted the application prior to the last guidance document from EFSA.

4.4.2.1 Two studies of four weeks duration with processed and unprocessed soybean 40-3-2, respectively , submitted by the applicant.

In the first of the two four weeks studies, Charles River CD rats of both sexes (10 animals/sex) were fed *ad libitum* a diet with 24.8% processed (dehulled, defatted and toasted) soybean material from either event 40-3-2, 61-67-1 (a non-commercial glyphosate-tolerant soybean transformed with plasmid PV-GMGT04) and A5403, a traditional soybean with the same genetic background as 40-3-2 (the conventional counterpart) (Hammond et al.,1996; 1993b). Based on the proximate analysis, the rodent diets were formulated to be comparable in total protein content and as similar as possible to the nutrient profile for the standard rat diet. An additional group of animals were fed a commercial rat diet (Purina Rodent Laboratory Rodent Chow) containing dehulled soybean meal. Body weights were recorded prior to randomisation and weekly for each animal. During the study the test animals were inspected twice daily. They appeared healthy and no mortality or adverse clinical signs were observed. At the end of the study, all test animals were sacrificed and necropsied. The diet did not influence feed consumption, body weights or organ weights of the rats (liver, testes, and kidneys). The few findings at the histopathological examinations at necropsy were of the same kind as are commonly observed in control animals of this rat strain in the testing laboratory. Moreover, the findings were randomly distributed among treatment groups. No adverse effects were observed in rats fed up to 24.8% processed glyphosate-tolerant soybean meal in the diet. The study concluded that processed soybean meal from glyphosate-tolerant soybeans is as safe as traditional soybean meal when fed to rats.

The second four weeks study submitted by the applicant had an experimental design very similar to the first study and also used Charles River CD rats of both sexes (Hammond et al., 1996; 1993). The study was conducted according to EPA GLP Standards, except that test material characterisation and stability measurements were not performed. Groups of CD rats (10/sex/group) were fed ad libitum 0, 5 or 10% (w/w) raw 40-3-2, raw 61-67-1 (a non-commercial glyphosate-tolerant soybean transformed with plasmid PV-GMGT04) and A5403, a traditional soybean with the same genetic background as 40-3-2 (i.e. the control) which were grown concurrently in the same field. These low inclusion rates were used since monogastric animals usually are not fed raw soybeans due to the presence of anti-nutritive

factors. Ruminants tolerate the raw soybean because the anti-nutrients are degraded by the rumen micro-flora.

Animals were observed for adverse signs and body weights were recorded weekly. The test animals appeared healthy, and the diet did not influence feed consumption, body weight, cumulative body weight gain, nor had any significant influence on absolute and relative organ weights (liver, testes, and kidneys) in relation to the conventional counterpart. At the end of the study, all test animals were sacrificed and necropsied. Organs were collected and weighed at the end of the study. The pancreas underwent histological examination. Test animals survived and appeared healthy and showed no treatment–related deaths or adverse signs of toxicity. No significant differences in body weight, body weight gain or in food consumption between the groups were observed. At the 5% dose of 40-3-2 a slightly increased relative kidney weight was observed, but not at the higher dose. Because the finding was independent of dose, it was not considered related to the treatment. Animals that received the higher dose of unprocessed soybean showed dark livers, this was likely related to the unprocessed soy and not to the genetic modification. Histological examination of the pancreas in all groups showed no macroscopic pathological lesions, but minimal to mild microscopic changes. The applicant concluded that the unprocessed meal from glyphosate–tolerant soybeans is as safe as meal from the parental soybeans.

4.4.2.2 Four week (30 day) toxicity study not submitted by the applicant

A transient mild histological alteration in the pancreas and a fast recovery has been reported in rats fed up to 30 days with a diet containing 18% soybean protein (Magaňa-Gómez et al., 2008). The products used in the study, commercial soy protein isolates (SPI) labeled as GM (SUPRO 500E) or Non-GM (SUPRO 500E IP) were purchased and GM or non-GM origins were confirmed by PCR. The soybean powder contained 5% Roundup Ready™ soybean. Unfortunately, in this study it is unclear whether the control diets used was based on soybean isogenic to soybean 40-3-2 or another type of commercially available non-GM soybean. It is also unclear whether the soybean products used have been appropriately processed before being included in the diet. Thus, it cannot be excluded that the transient alterations reported could have been the result of non-controlled levels of anti-nutrients in the diet.

4.4.2.3 Assessment of Immunotoxicity

The applicant submitted a 15-week rat sub-chronic feeding study with heat-treated soybean meal in female brown Norway rats and female B10A mice (Teshima et al., 2000). The studies were designed to compare the feeding value of a line of genetically modified glyphosate-tolerant soybeans (GM soybeans) to that of closely-related and one-parent same cultivar (non-GM soybeans). The aim was to study potential effects on the immune system. The heat-treated soybean meal was incorporated at a rate of 30% in the rat and mice feed

produced from soybean 40-3-2 in the test group and from a closely related conventional non-GM soybean in the control group. The histopathology of the thymus, liver, spleen, mesenteric lymph nodes, Peyer's patches and the small intestine, and the production of soybean-specific IgE and IgG antibodies in the sera were compared. Growth, food consumption, liver and spleen weight and the histopathology of immune-related organs showed no significant treatment-related differences between animals fed soybean 40-3-2 and non-GM lines. The presence of soybean specific IgE was not detected in the sera of either treatment group. Also the increase in soybean-specific IgG was identical in the GM and non-GM groups. No sign of immunotoxicity was found in GM-soybean fed rats or mice. Teshima et al. (2000) concluded that soybean 40-3-2 not was more antigenic or immunogenic than traditional soybeans.

The potential effect of a diet composed of genetically modified feed components on the selected immune parameters was investigated in pigs, cattle, and poultry (Bednarek et al (2013)). The aim of the study was to evaluate the immunological responses in domestic animals when fed genetically modified (GM) insect-resistant corn (MON810) expressing toxin protein of Bacillus thuringiensis and/or glyphosate-tolerant soybean meal (Roundup Ready 40-3-2). The study was conducted on 60 pigs (36 fatteners and 24 sows), 20 calves, 40 broilers, and 40 laying hens. Each species was divided into four basic nutritional groups: group I (control) - conventional feed, group II: feed consisted of GM soybean meal and non-modified corn, group III: non-modified soybean meal and GM corn, group IV: GM soybean meal and GM corn. Moreover, in the experiment on fatteners two additional groups were included: group V: animals fed both conventional soybean meal and bruised grain, and group VI: GM soybean meal and conventional bruised grain.

For pigs, poultry, and cattle the results of study did not reveal any significant changes in: peripheral WBC, leukogram (the percentage of differentiation of leukocyte subpopulations including LYM, PMNL, and MID), lymphocyte immunophenotyping with a detailed classification of CD3, CD4, and CD8 (CD8a) positive cell subsets. WC4 positive cell subpopulation representing bovine B peripheral blood lymphocytes was also studied in cattle. No significant differences were reported. The analysis of these WBC components confirmed the lack of GM feed influence on the cellular immune response in the investigated animals. The authors concluded that results indicate that meal from Roundup Ready 40-3-2 and insect-resistant MON810 maize did not affect the cellular and humoral immunity of fattened pigs, poultry, and cattle.

4.4.2.4 Sub-chronic feeding studies in rats with processed and heat-treated soybean 40-3-2

The applicant submitted a 13-weeks (90-day) feeding study in Sprague-Dawley rats fed ad libitum diets with processed soybean 40-3-2 meal or meal from a conventional soybean (Zhu et al., 2004). The rats were fed four experimental corn-based diets containing 60%

conventional soybean meal, a mixture of 30% conventional and 30% soybean 40-3-2 meal, 60% or 90% soybean 40-3-2 meal. All diets were adjusted to an identical nutrient level except the 90% soybean 40-3-2 diet. There were 10 females and 10 males per dietary treatment. During the first week, rats of both sexes fed 90% soybean 40-3-2 meal exhibited a deviation in feed intake and body weight, apparently due to the high protein levels of the diet and not to the exposure to soybean 40-3-2. This effect on feed intake and body weight gain was not observed later on in the study. The study demonstrated that the two types of soybean meal prepared from GM herbicide-tolerant (RR) and nearly isogenic conventional soybeans were comparable in composition and nutritional value for Sprague-Dawley rats. Moreover, no evidence of any pathological signs of the soybean 40-3-2 soybean meal was found even at 90% in the diet (two or three times greater than normal). No biological significant differences in gross necropsy findings, haematology or urinalysis parameters between rats fed processed soybean 40-3-2 and conventional soybean meal were found. Moreover, the *cp4 epsps* gene specific constructs from soybean 40-3-2 meal or lec gene from conventional soybean meal DNA could not be detected in investigated muscle samples in the rats. Zhu et al (2004) concluded that soybean 40-3-2 meal fed during a 13-week period did not produce any adverse effects in rats even at a proportion as high as 90% in the diet.

4.4.2.5 Chronic feeding studies

In two long-term studies over 1 year (52 weeks) and 2 years (104 weeks), respectively, Japanese investigators fed F344 DuCrj rats diets containing either 30% of a powder of processed soybean 40-3-2 or of the non-GM soybean conventional counterpart, having a similar genetic background to soybean 40-3-2, or a basal diet (CE-2) (Sakamoto et al., 2007, 2008). When the three groups were compared, some statistically significant differences in animal growth, food intake, serum biochemical parameters and histological findings were noted, in particular between rats fed the two types of soybean diet (with GM and non-GM soybean) and the rats fed the basal diet. However, body weight and food intake were similar for the rats fed soybean 40-3-2 and conventional soybean. Gross necropsy findings, haematological and serum biochemical parameters, organ weights, and microscopic findings were comparable between rats fed soybean 40-3-2 and conventional soybean. In the 2-year study, the histopathological investigations did not reveal any increase in the incidence, nor in any specific type of non-neoplastic or neoplastic lesions in the GM soybean-exposed group of both sexes. The investigators concluded that the long-term effects of soybean 40-3-2 are not different from the long-term effects of non-GM soybeans.

4.4.2.6 Studies testing effects on reproductive performance

Brake and Evenson (2004) performed one sub-chronic development (87 days) and one multigenerational (4 generations) feeding study with C57Bl/6J mice fed transgenic (40-3-2) or non-transgenic soybean meal as 21.35% of the diet. Possible toxic effects were studied

with a mammalian testis model. Pregnant mice were fed a transgenic soybean or a non-transgenic (conventional) diet through gestation and lactation. After weaning, the young male mice were maintained on the respective diets. At 8, 16, 26, 32, 63 and 87 days after birth, three male mice and an adult reference mouse were killed, the testes surgically removed, and the cell populations measured by flow cytometry. Another multi-generational study was conducted in the same manner. The results showed that the transgenic foodstuffs had no effect on macromolecular synthesis or cell growth and differentiation as evidenced by no differences in the percentages of testicular cell populations (haploid, diploid, and tetraploid) between the transgenic soybean-fed mice and those fed the conventional diet. Additionally, there were no differences in litter sizes and body weights of the two groups. The investigators concluded that the transgenic soybean diet had no negative effect on fetal, postnatal, pubertal or adult testicular development.

Malatesta and co-workers, summarised their result of studies in which progeny of Swiss mice were fed diets contained 14% soybean 40-3-2 or wild type soybean during pregnancy and/or for 1, 2, 5, 8 or 24 months after weaning. (Malatesta et al., 2002a, 2002b, 2003, 2005, 2008; Vecchio et al., 2004). In most of these studies only female mice were used. Growth was comparable in animals receiving the two types of diets, and no macroscopic alterations or pathological lesions were found. The investigators reported differences in transcriptional activity, revealed as alterations in staining characteristics of chromatin-associated elements in cell nuclei. It should be noted that the investigators concluded from only three animals per treatment and gave no information on the natural variability in the specific histocytochemical endpoints analysed. It was suggested that the altered staining characteristics indicated that feeding diets containing GM soybean may be associated with reversible changes in nucleic transcriptional activity, possibly as a consequence of exposure to residues of glyphosate, differences in phytoestrogen content between the diets, the genetic modification in soybean 40-3-2, or a combination of these. However, the experimental designs of the studies and their evaluation can be criticised. The studies did not provide detailed account of the origin and characteristics of the control soybeans used, or whether the soybeans were processed or not, neither were the levels of soybean bioactive constituents in the two diets stated. In addition, it is noted that in these studies particular biological phenomena were examined but not those parameters which are normally regarded as indicative of specific organ toxicity. Also the statistical evaluation of the data has been criticised.

4.4.3 Studies on Allergenicity

4.4.3.1 Assessment of allergenicity of the transgenic protein

The applicant has assessed the allergenic potential of the CP4 EPSPS protein by bioinformatic comparison of the amino acid sequence of the CP4 EPSPS protein produced in 40-3-2 with known allergen database sequences, as well as the evaluation of the stability of the protein in an in vitro gastric digestion model.

They claim that the gene encoding the CP4 EPSPS protein was not obtained from a source known to be allergenic. The CP4 EPSPS protein was initially obtained from the naturally occurring soil-borne and plant-symbiotic bacterium *Agrobacterium* sp. strain CP4 (Padgette et al., 1993). To date, no findings of allergy to Agrobacterium have been reported and this *Agrobacterium* does not belong to one of the eight common food categories known to cause >90% of all food allergic reactions (Hefle et al., 1996). The bioinformatic analyses were conducted to assess the potential for allergenicity of the CP4 EPSPS protein sequence (McCoy and Silvanovich, 2003). The allergen, gliadin and glutenin sequences database (AD4) was assembled from publicly available databases (GenBank, EMBL, PIR, NRL3D version of RCSB PDB and SwissProt) and from current literature. The amino acid sequence of the CP4 EPSPS protein was compared to all sequences in the databases with the FASTA sequence alignment tool. The extent of each similarity was evaluated by visual inspection of the alignment, the calculated percent identity and the E score for that alignment. Additionally, the CP4 EPSPS amino acid sequence was also screened against the allergen database with an algorithm that scans for a window of eight linearly contiguous amino acids. The presence of such identities might indicate potentially cross-reactive allergenic epitopes. The results of this bioinformatics search indicate that the CP4 EPSPS protein shares no structurally significant sequence similarity to sequences within the allergen databases and no immunologically significant sequence similarity to protein associated with IgE-mediated allergies or to the proteins associated with celiac disease.

This is in agreement with the assessment of the allergenic potential of the CP4 EPSPS protein conducted by UK-ACNFP (1995) which showed that it is unlikely to be an IgE-dependent allergen since i) the *cp4 epsps* gene was taken from a source not known to be allergenic, and ii) the molecular weight of the protein and its glycosylation characteristics and acid lability are not indicative of an increased risk of allergenicity. In addition, a bioinformatics-supported comparison of the amino acid sequence of the CP4 EPSPS protein with the sequences of known allergens, gliadins, and glutenins (which included an updated analysis with published databases), identified no similarities which would cause concern.

European and Asian patients allergic to soybean and/or other foods do not express IgE that specifically bind the purified CP4 EPSPS protein (Chang et al., 2003; Batista et al., 2005; Kim et al., 2006a, 2006b; Hoff et al., 2007). The purified CP4 EPSPS enzyme also did not result in pronounced change in histamine release or cytokine production in sensitised peritoneal mast cells or unsensitised but antisera-labelled mast cells cultivated in vitro (Chang et al., 2003). It is considered that these studies further confirm that the newly expressed CP4 EPSPS protein is unlikely to be allergenic.

4.4.3.2 Assessment of the allergenicity of the whole GM plant

In the submitted dossier the applicant has assessed the allergenicity of the whole GM plant as follows: Soybean is known to cause food allergies in certain individuals (Burks et al.,

1988). Therefore, an assessment of the endogenous allergens in 40-3-2 and traditional soybean has been conducted, with sera from patients sensitive to soybean protein (Burks and Fuchs, 1995). The purpose of the study was to qualitatively and quantitatively compare the endogenous allergens in 40-3-2 to A5403, a traditional soybean with the same genetic background as 40-3-2, and to three commercially available traditional soybean varieties. As expected, the analysis of the protein extracts prepared from 40-3-2 revealed that both the composition and the quantity of proteins detected by immunoblotting were indistinguishable from the results produced with A5403 (the control) and three traditional soybean varieties, demonstrating that the production of the CP4 EPSPS protein in 40-3-2 does not cause any change in the composition of the allergenic proteins endogenous to soybean. Additionally, more recent publications have confirmed the conclusions reported by (Burks and Fuchs, 1995) for 40-3-2. Namely, that none of the individuals undergoing allergenicity tests reacted differently to 40-3-2 than to traditional soybean samples (Batista et al., 2005). Moreover, a lack of detectable allergenicity towards the produced CP4 EPSPS protein was reported (Batista et al., 2005; Chang et al., 2003). The applicant has therefore concluded that 40-3-2 is as safe as traditional soybeans in terms of allergenic potential.

Allergenicity of the soybean could be increased as an unintended effect of the random insertion of the transgene in the genome of the recipient, e.g. through qualitative or quantitative modifications of the expression of endogenous proteins. UK-ACNFP (1995) noted that soybeans are known to be allergenic for certain individuals. However, studies supplied in the original notification under Directive 90/220/EEC (Burks and Fuchs, 1995), allowed the conclusion that the levels of known allergenic proteins in soybean 40-3-2 does not differ from the levels in non-GM soybeans. The results of these initial pre-marketing studies have recently been confirmed after the product has been on the market for some time. With two-dimensional gel electrophoresis followed by peptide tandem mass spectrometry to identify soybean proteins, and Western analysis to evaluate the IgE response of soybean allergic individuals, Batista et al. (2007) were able to show that none of the five soybean-allergic individuals tested reacted differently to soybean 40-3-2 and its appropriate conventional counterpart. Similarly, several other investigations based on blood/sera of soybean allergic patients (from Denmark, Korea, Portugal) or on skin prick tests have found no difference in allergenic potential of extracts of soybean 40-3-2 and extracts of non-GM soybeans (Park et al., 2001; Sten et al., 2004; Batista et al., 2005; Kim et al., 2006a, 2006b; Hoff et al., 2007). Furthermore, another study (Hoff et al. 2007) did not observe cross-reactivity between CP4 EPSPS and known allergens including the mite allergen “Der f 2” with sera of patients allergic to certain foods and mites.

4.4.3.3 Assessment of allergenicity of proteins from the GM plant

Allergenicity of the soybean could be increased as an unintended effect of the random insertion of the transgene in the genome of the recipient, e.g. through qualitative or quantitative modifications of the expression of endogenous proteins. However, given that no

biologically relevant agronomic or compositional changes have been identified in soybean with the exception of the introduced traits, no increased IgE mediated allergenicity is anticipated for soybean.

4.4.4 Assessment of Adjuvanticity

According to the EFSA Scientific Opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed from GM plants (EFSA 2010c), adjuvants are substances that, when co-administered with an antigen increases the immune response to that antigen and therefore might increase the allergic response. Adjuvanticity has not been routinely considered in the assessment of allergenicity of GMOs.

In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the possible role of these proteins as adjuvants should be considered. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity.

"Bystander sensitisation" can occur when an adjuvant in food, or an immune response against a food antigen, results in an increased permeability of the intestinal epithelium for other components in food. Previously it was assumed that the epithelial cells of the intestine were permanently "glued together" by the so-called "tight junctions". More recent knowledge shows that these complex protein structures are dynamic and can be opened up by different stimuli.

Both in vitro and in vivo experiments have demonstrated that when an IgG response which can result in a complement activation (among other) is not balanced by an IgA response, the epithelial barrier can become leaky and unwanted proteins are able to enter the body (bystander-penetration) and possibly lead to allergic sensitisation (Brandtzaeg & Tolo, 1977; Lim & Rowley, 1982).

4.5 Nutritional assessment of GM food and feed

Compositional analyses of soybean indicate nutritional equivalence to the non-GM control soybean with comparable genetic background and to the published range of values in the literature. The nutritional equivalence between soybean and non-GM control soybean has been further shown by the results of broiler, catfish, dairy cows, pigs and quail feeding studies see chapter 5.6.2.

4.5.1 Intake information/exposure assessment

The human soybean oil consumption in Europe was calculated at 6.3-7.0 g/person/day, based on FAO Statistics from 1997 to 2001. Assuming that 54% of the soybean oil was derived from soybean 40-3-2, the estimated average exposure of the European consumer to products of soybean 40-3-2 would be approximately 3.4-3.7 g/person/ day (Technical dossier).

Soy beans and their products are little used in the average Norwegian diet, with the exception of vegans and those with milk allergies.

In table 4.5.1-1 the mean intake of soy protein/day for an adult person in Norway eating either a vegan menu or a milk free diet are presented (Engeset & Lillegaard, 2014, unpublished results). The calculations were based on week menus. For the vegan menu a person who has previously eaten meat and is looking for meat substitutes like soy burgers and sausages were envisioned. In the milk free diet a 7 day week menu was composed where milk products were replaced with soy products. Both menus are included in appendix II.

Table 4.5.1-1. Mean intake of soy products and soy protein for adult persons with milk allergy and vegans with high preference for soy products.

Diet	MJ/day (mean)	Gram soy products/day (mean)	Gram soy protein/day (mean)
Milk allergy	9,7	538	19
Vegan	10,1	865	35

Average estimated energy requirement for children in different age groups, based on The Nordic Nutrition Recommendations (NNR), was used to adjust the numbers in table 4.5.1-1 according to age to give an estimate of how much soy protein children may consume if on the given diets (Table 4.5.1-2). We assumed that milk in coffee/tea in the menus is consumed as milk by the children.

Table 4.5.1-2. Estimated intake of soy products and soy protein for children in different age groups, with milk allergy and vegans, and with high preference for soy products.

Diet	Estimated energy requirement MJ/day[1]	Gram soy products/day	Gram soy protein/day
Milk allergy			
2-5 year	5,3	294	10
6-9 year	6,9	383	14
10-13 year (girls)[2]	8,6	477	17
14-17 year (boys) [2]	11,8	655	23
Vegan			
2-5 year	5,3	454	18
6-9 year	6,9	591	24
10-13 year (girls) [2]	8,6	737	30
14-17 year (boys) [2]	11,8	1011	41

1 Based on Nordic Nutrition Recommendations 2012
2 Boys 10-13 years and girls 14-17 years will have approximately the same consumption as adults; estimated energy requirement of 9,3 and 9,8 respectively.

Around 90% of the soybean defatted protein meal worldwide goes to animal feed, while there is limited use of soybean oil in feed. The applicant calculated, based on data from 2006, that the maximum inclusion levels (% of the diet) of soybean 40-3-2 meal in the EU would be 21% for broiler, 18% for pigs and 12% for dairy cattle (Technical dossier).

In Norway, almost 1.5 mill tons of fish feed was produced in 2012 and soybean concentrate is one important protein source in salmon feeds (Directorate of Fisheries, Biomass statistics 2013). The average inclusion level of soybean protein concentrate (SPC) in feed to Atlantic salmon is 25%, total SPC used for fish feed production in 2013 was calculated to be approximately 37.500 ton (Annual Sustainability report, Skretting, 2013).

Assuming that 100% of the SPC was derived from soybean 40-3-2, the estimated average exposure of Atlantic salmon (post smolt, 200 g) to products of soybean 40-3-2 would be approximately 2 g/fish/day (assuming 3% growth per day and feed conversion ratio of 1).

Norwegian surveillance data show that imported SPC intended for feed production only contains trace amounts of GMO (*e.g* below 0.9%) (Spilsberg et al 2014). The DNA specific targets that are included in the GMO methodology are 35S promoter (p35S), *Agrobacterium* nopalin synthase terminator (tNOS), *ctp2-cp4epsps*, the *bar* gene from *Streptomyces hygroscopicus* and the *pat* gene from *Streptomyces viridichromogenes*.

4.5.2 Nutritional assessment of feed derived from the GM plant

4.5.2.1 Applicant's data for nutritional assessment

The applicant has carried out feeding studies with soybean 40-3-2 and corresponding control diets in chickens, catfish, dairy cows, pigs and quail.

Six weeks feeding study with soybean on broilers

Broilers were fed starter diets containing 32.9 % processed (dehulled, defatted and toasted) soybean meal (soybeans 40-3-2, a non-commercial glyphosate-tolerant soybean (61-67-1) or a commercial soybean (A5403) with the same genetic background as 40-3-2 from day 0-21 (Hammond et al. 1996). A grower/finisher diet containing 26.6% soybean meal was fed from day 22-42 when the study was terminated. In these 42 days, the broilers reached a marked weight of 2 kg. All diets met or exceeded National Research Council (NRC) requirements for poultry. For the starter period (days 0-21), there were no differences in body weight and live weight gain or percent live birds for any of the groups. For the 22-42 day period and the cumulative study period (days 0-42), there were no statistically significant differences between groups for body weight, live weight gain, feed intake, F/G or percent live birds. The experimental diets had no influence on feed intake, weight gain, feed conversion, and liveability (percent live rats; survival rate). There were also no significant difference in the performance parameters investigated (breast muscle weight and abdominal fat pad weight; in both cases total weight and percent of body weight) between broilers fed diets with soybean 40-3-2 and broilers fed its conventional counterpart.

Additional information on broilers is available from a small feeding study in which the birds were given a diet with 24–25 % soybean meal (Deaville and Maddison, 2005). The broilers

fed soybean 40-3-2 had as high feed intake, growth and feed conversion ratio as broilers fed control soybean.

Five day dietary study with quail fed unprocessed soybeans

The dietary study was conducted to compare the wholesomeness of 40-3-2 and traditional soybean (Technical dossier Part I). Young quail were fed 20 % raw soybean meal for five days. Thirty bobwhite chicks of mixed sex were assigned by indiscriminate draw to each treatment and housed in groups with ten birds/pen. Each control and treatment group was fed the test diet for five days and then switched to basal (unsupplemented) diets for the last three days of the study. Food and water were provided ad libitum. Food consumption was recorded daily for each pen (days 1-5) and average food consumption/pen was recorded for days 6 to 8. Food consumption is an estimate due to the unavoidable wastage by the birds. Individual body weights were recorded at study initiation, on study day 5 and at study termination.

No treatment related mortality or differences in food consumption, body weight gain or behavior occurred between birds fed 20% (w/w) glyphosate-tolerant soybean meal and birds fed traditional soybean meal or basal diet only. This study is of short duration, and therefore of limit value in nutritional assessment.

Ten week feeding study with catfish fed processed soybeans

The feeding study was performed on 300 fingerling channel catfish (*Ictalurus punctatus*) of mixed sex with 5 tanks per treatment (n=5) and with three diets containing either soybeans 40-3-2, a non-commercial glyphosate-tolerant soybean (61-67-1) or a commercial soybean (A5403) (Hammond et al. 1996).The study was over ten weeks with diets containing processed meal (45–47 % w/w) (Hammond et al. 1996). There was no statistically significant difference in survival, feed conversion ratio, and percentage weight gain between the groups receiving diets based on control soybean meal and soybean 40-3-2 meal. Although fish receiving the diet with soybean 40-3-2 meal consumed slightly less feed than fish fed a diet with the control soybean meal, this did not influence body composition data. There were no differences in moisture, protein, fat or ash content in the fillet of the catfish regardless of dietary treatment.

Four week feeding study with cows fed raw soybeans

Holstein dairy cows were fed either raw soybean 40-3-2, soybean 61-67-1 (non-commercial glyphosate-tolerant) or soybean A5403 (commercial with the same genetic background as 40-3-2, as control) (Hammond et al. 1996). The soybeans were incorporated in the mixed diet ration at a concentration of 10% (w/w dry matter basis) which represents an upper limit for incorporation of raw soybeans into mixed cow diets.

Diets were formulated to meet or exceed nutrient recommendations of the 1989 National Research Council (NRC) and provided similar quantities of crude protein, net energy, lipid, fibre, major minerals and vitamins, while maintaining equal proportions of soybeans on a dry matter basis.

Thirty-six multiparous Holstein dairy cows between 93 and 196 days of lactation were assigned to one of two blocks based on availability. The days of lactation for cows in the first block ranged from 122 to 196 days and for the second block, 93 to 135 days. Within blocks, cows were randomly assigned to groups (5-6 cows) fed 40-3-2, 61-67-1 or A5403 raw soybeans. Cows were individually identified and housed in a tie-stall barn and released into an exercise lot prior to each milking.

Observations for overall health were recorded twice daily. Cows were weighed on treatment days -1, 0, 28 and 29 and their body condition was scored at the same days.

During the fourth week urine and faces were collected, and analysed for dry matter and nitrogen balance. On the last day of the study rumen fluid was collected and analysed for volatile fatty acids and ruminal ammonia.

Animal health was good throughout the study. There were no statistical differences in last-square means for milk production, milk fat, protein, lactose, somatic cell count, dry matter intake, net energy intake, fat corrected milk (FCM)/net energy level (NEL), or body weight change. There was a small (2.5-2.7 kg/day) but statistically significant increase in 3.5% fat corrected milk for cows fed both 40-3-2 and 61-67-1. This higher production is consistent with a non-significant increase in net energy intake of glyphosate-tolerant soybeans resulting in similar ratios of FCM production to NEL intake. There were no statistically significant differences in least-square means for dry matter intake, nitrogen intake, dry matter digestibility and milk, urine, fecal, absorbed, retained or productive nitrogen. There were no statistically significant differences in least-square means for ruminant fatty acids (acetate, propionate, isobutyrate, isovalerate, valerate and acetate/propionate) or ruminal ammonia nitrogen.

Feeding study on pigs with soybean meal

The nutritional value of 40-3-2 was further assessed in a study where 100 growing–finishing pigs were fed soybean meal diets containing 14-24% (depending on age of the animals) of dehulled soybean meal from either soybean 40-3-2 or traditional soybean meal (Cromwell et al. 2002). Soybean 40-3-2 was treated with Roundup herbicide. Rate and efficiency of weight gain, scanned backfat and longissimus area, and calculated carcass lean percentage were not significantly different ($P > 0.05$) for pigs fed diets containing conventional or Roundup Ready soybean meal. Responses to the type of soybean meal were similar for the two sexes with no evidence of a diet x sex interaction for any of the traits. In most instances, carcass traits of the barrows were similar for the two types of soybean meal. Longissimus muscle

samples from barrows fed conventional soybean meal tended to have less fat than those fed Roundup Ready soybean meal (P = 0.06), but the contents of water, protein, and ash were similar. Sensory scores of cooked longissimus muscles were not influenced (P > 0.05) by diet. Cromwell et al. (2002) concluded that soybean 40-3-2 meal is equivalent in composition and nutritional value to traditional soybean meal for growing-finishing pigs.

4.5.2.2 Other peer reviewed feeding studies

Feeding studies performed on Atlantic salmon with meal from Roundup Ready soybeans

In the study by Sanden et al. (2006) both soybean 40-3-2 and maize MON 810 was used in the feed for salmon. In two of the feeds standard fishmeal was replaced by soybean 40-3-2 or a commercial non-GM soybean at a level of 12.5 % of the total diet. Each diet was fed to fish in triplicate tanks. The experiment was conducted over 8 months, during which the fish grew from 0.2 g to 101-116 g.

There was no significant effect of diet on the intestinal indices, nor were histological changes observed in the pyloric caeca or mid intestine. In fish fed the non-GM soybean diet, a significantly higher ($p<0.05$) cell proliferation response (PCNA) was observed in the distal intestine compared with fish fed the GM soybean diet, the fishmeal-based diet and the maize-based diets ($p<0.05$). The authors suggests that differences in distal intestinal cell proliferation may relate to different content of ant nutrients in the diets. According to the authors, feed from GM- or non-GM soybeans, at an inclusion level of 12.5 %, were as safe as commercial available non-GM feed.

Sissener et al. (2009 a, b) performed a 7-month feeding trial with diets containing 25 % meal of soybean 40-3-2 or its conventional counterpart in Atlantic salmon (Salmo salar) in order to study growth, body composition, organ development, intestinal changes, haematological parameters, clinical chemistry and lysozyme levels, and stress response. Of the many parameters studied, only mid-intestine being smaller, plasma triacylglycerol levels being higher, and the mucosal fold height in the distal intestine (one sampling time of three) and mucosal fold fusion was more pronounced in fish supplied the diet with GM soybean. No other diet-related morphological differences were found in any organs, and there was no difference in stress response.

Proteomic profiling of liver cells from these salmons only identified minor differences in liver protein synthesis between fish fed GM and non-GM soybean (Sissener et al., 2010a).

Sanden et al. (2011) followed the fate of soybean DNA in the intestinal tract of the salmon. Transgenic DNA was not detected in any of the analysed intestinal organs but the multi-copy rubisco gene was found in all segments of the intestine. Feed restriction gradually cleared DNA within five days. Re-feeding revealed DNA within two hours. Thus, it seems as feeding status regulates the appearance of DNA in various intestinal segments. The investigators

concluded that it appears as inclusions levels of 25 % GM soybean in the fish diet does not cause any adverse effects of importance on organ morphology or stress response compared with non-GM soybean.

The paper by Bakke-McKellep et al. (2007) reports the second and final part of an experiment aiming to study physiological and health-related effects of genetically modified (GM) soybean meal (SBM) type Roundup Ready soybean (40-3-2) in diets for post-smolt Atlantic salmon. For 3 months salmon were fed diets containing 172 g/kg full-fat SBM from (GM-soy) or an unmodified, non- isogenic line (nGM-soy), or a reference diet with fishmeal as the sole protein source (FM). Slight differences in anti-nutrient levels were observed between the GM and nGM-soy. Histological changes were observed only in the distal intestine of the soy-fed fish. The incidence of moderate inflammation was higher in the GM-soy group (9 of 10 sampled fish) compared with the nGM-soy group (7 of 10). However, no differences in the concomitant decreases in activities of digestive enzymes located in the brush border (leucine aminopeptidase and maltase) and apical cytoplasm (acid phosphatase) of enterocytes or in the number of major histocompatibility complex class II+ cells, lysozyme activity, or total IgM of the distal intestine were observed.

GM compared with non-GM-soy fed fish had higher head kidney lysozyme (11,856 vs. 10,456 units/g tissue) and a tendency towards higher acid phosphatase (0.45 vs. 0.39 micromol/h/kg body mass in whole tissue) activities, respectively. Plasma insulin and thyroxin levels, and hepatic fructose-1,6-bisphosphatase and ethoxyresorufin-O-deethylase activities were not significantly affected.

According to the authors it is not possible to conclude whether the differences in responses to GM-soy were due to the genetic modification or to differences in soy cultivars in the soy-containing diets. Results from studies with non-modified, parental line soybeans as the control group are necessary to evaluate whether genetic modification of soybeans in diets poses any risk to farmed Atlantic salmon.

Bakke-McKellep et al. (2008): Physiological and health related responses to dietary inclusion of genetically modified (GM) full-fat soybean meal (Roundup Ready 40-3-2) and maize (MON810 Bt-maize; GM-maize), as well as non-parental, untransformed lines (nGM-soy and nGM-maize D2), were evaluated in farmed Atlantic salmon (*Salmo salar* L.) parr during the first 8 months of feeding. Significant effects of dietary GM presence were only found in intestinal Na+-dependent d-glucose uptake and SGLT1 protein level in the region pyloric caeca in which the highest values were found in the GM-soy, intermediate in the non GM-soy, and lowest in the standard FM fed groups. Data from this study confirm that GM soybeans (40-3-2) and maize (MON810) at inclusion levels of about 6% appear to be as safe as commercially available nGM soy and maize in diets for Atlantic salmon parr. Results from studies with higher inclusion levels and with non-modified, isogenic or near-isogenic parental lines as control groups are pending.

Feeding studies performed on pigs.

Swiatkiewicz et al (2011a) fed pigs with diets containing meal of soybean 40-3-2 and/or the conventional counterpart. The progeny of all sows was also included in the experiment. Feed utilisation, body weight, and carcass yield was not different between soybean 40-3-2 and its conventional counterpart. Type of soybean in the diet also did not influence the quality and chemical composition of the meat. The dietary treatments had no influence on colour parameters of the loin meat, whereas some differences were noted in the neck muscle, possibly due to the natural heterogeneity of this primal cut. DNA fragments specific for soybean 40-3-2 could be identified in the content of the stomach and duodenum but not further down in the gastrointestinal tract, and in various tissues and in blood.

Swiątkiewicz et al. (2013) investigated the effect of Roundup Ready 40-3-2 soybean meal and Bt maize MON810 on sow performance and haematological indices, including parameters of erythrocytes, leukocytes, and trombocytes. The experiment were carried out on 24 sows and their progeny and also included evaluation of the piglets rearing indices. All feed mixtures were isonitrogenous and isoenergetic, however differed in presence or absence of genetically modified soybean meal 40-3-2 (4% for pregnant and 14% for lactating sows). Conventional soybeans were used as control. The experiment was conducted on 24 sows mated with boar. Body weight was controlled at mating (206.5-225 kg), at the 100th d of pregnancy (248.0-267.7 kg), after farrowing (238.7-259.3), and after the weaning of piglets (28th d of lactation) (228.8-246.7 kg). During lactation sows lost weight from 9.9 – 12.6 kg.

The effect of soybean 40-3-2 meal on sows performance and haematological indices, were evaluated in the experiment. Born piglets were allotted to the same group as their mothers. Since the 7th d of age, the piglets were fed diets differing in presence or absence of genetically modified soybean meal MON-40-3-2 (26% in diet) and/or maize MON810 (10% in diet).

The study showed that feeding pregnant and lactating sows with mixtures containing genetically modified soybean 40-3-2 did not significantly affect their reproductive characteristics and offspring performance. There was no effect of dietary treatment on haematological indices. Results of the study indicate that feeding genetically modified soybean 40-3-2 meal to pregnant and lactating sows did not significantly affect their condition during reproductive cycle and the quality of their litters. Piglets rearing indices also remained unchanged. Fragments of transgenic DNA typical for genetically modified soybean was not detected in sows' blood samples.

Feeding study with soybean on broilers

Świątkiewicz et al. (2010a) fed Ross 308 broilers diets with 32–39 % soybean meal from soybean 40-3-2 or a commercial non-GM soybean variety. Feed intake, growth parameters, and mortality were not different in the two groups, and there were no statistically significant

differences found in carcass parameters, organ weights, and chemical composition of the breast muscles analysed after slaughter. The pH and water holding capacity values of breast and thigh muscles indicated no statistically significant differences between broilers fed diets containing soybean 40-3-2 and birds fed diets with meal of the commercial non-GM soybean variety (Stadnik et al. 2011a). Furthermore, studies in Bovans Brown laying hens by the same investigators showed that the laying performance, digestibility of nutrients and egg quality did not differ between hens that received meal of soybean 40-3-2 and hens that received meal of commercial non-GM soybean varieties in the diet (Swiatkiewicz et al., 2010b). Recombinant DNA was not detected in internal organs, blood, muscles, excreta and eggs of examined birds.

Possible GM-DNA transfer from GM-feed to birds and animals

Sieradzki et al (2013) have performed a study in order to assess the possibility of GM-DNA transfer from feed containing soybean 40-3-2 to animal tissues, gut bacterial flora, food of animal origin, and the fate of GM DNA in the animal digestive tract. The experiment was carried out on broilers, laying hens, pigs and calves. All animals were divided into four groups: I-control group (non-modified feed), II-GM soybean group (non-modified maize, 40-3-2 soybean), III-GM maize group (MON810 maize, non-modified soybean), and IV-GM maize and soybean group (MON810 maize, 40-3-2 soybean). Samples of blood, organs, tissues, digesta from the gastrointestinal tract, and eggs were analysed for the presence of plant species specific genes, and transgenic sequences of CaMV 35S promoter and NOS terminator. PCR amplifications of these GM sequences were conducted to investigate the GM DNA transfer from feed to animal tissues and bacterial gut flora. In none of the analysed samples of blood, organs, tissues, eggs, excreta and bacterial DNA were plant reference genes or GM DNA found.

A GM crop diet did not affect bacterial gut flora as regards diversity of bacteria species, quantity of particular bacteria species in the animal gut, or incorporation of transgenic DNA to the bacteria genome. The authors concluded that MON810 maize and 40-3-2 soybean used for animal feeding are substantially equivalent to their conventional counterparts. Genetically modified DNA from MON810 maize and 40-3-2 soybean is digested in the same way as plant DNA, with no probability of being transferred to animal tissues or gut bacterial flora.

4.6 Conclusion

Subchronic feeding studies on the glyphosate-tolerant soybean 40-3-2 in rats, as well as whole food feeding studies on broilers, quails, cows, pigs, piglets, catfish and Atlantic salmon have not indicated any adverse effects. The CP4 EPSPS protein does not show sequence resemblance to known toxins or IgE allergens, nor has it been reported to cause IgE mediated allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that soybean 40-3-2 is nutritionally equivalent and as safe as conventional soybean.

5 Environmental risk assessment

Considering the scope of the application EFSA/GMO/RX/40-3-2, the environmental risk assessment is concerned with the accidental release into the environment of viable soybean 40-3-2 seeds during transport and/or processing, and with indirect exposure through ingestion by animals and their manure and faeces leading to exposure of the gastrointestinal tract and soil microorganisms.

5.1 Unintended effects on plant fitness due to the genetic modification

Cultivated soybean, Glycine max (L.) Merr., is a member of the genus *Glycine* and belongs to the Fabaceae (Leguminosae) family. Soybean is an annual, subtropical plant, native to eastern Asia (OECD 2000). The crop is however grown over a wide range of ecological zones, ranging from the tropics to the temperate zones (Acquaah 2012). The major worldwide soybean producers are China, the United States, Brazil and Argentina (FAOSTAT 2013). In Europe, soybean is mainly cultivated in Ukraine, the Russian Federation, Italy, France and Romania. There is no cultivation of soybean in Norway.

Despite accidental seed dispersal and extensive cultivation in many countries, seed-mediated establishment and survival of soybean outside cultivation or on disturbed land is rare (OECD 2000). Establishment of feral soybean populations has never been observed in Europe. Soybean volunteers are rare throughout the world and do not effectively compete with the succeeding crop or primary colonisers (OECD 2000).

Soybean is a highly domesticated crop and generally unable to survive in the environment without management intervention (Lu 2005). The soybean plant is not weedy in character. As for all domesticated crops, soybean has been selected against seed shattering to reduce yield losses during harvesting. Cultivated soybean seeds rarely display any dormancy characteristics and have poor seed survivability in soils (OECD 2000). Due to low frost tolerance, susceptibility to plant pathogens, rotting and germination, the seeds will normally not survive during the winter (Owen 2005). The soybean seeds need a minimum soil temperature of 10 °C to germinate and the seedlings are sensitive to low temperatures (OECD 2000; Bramlage et al. 1978). Soybean is a quantitative short-day plant that needs short days for induction of flowering, and the growing season in Norway is too short for the soybean plant to reach full maturity. Potential soybean plants resulting from accidental

release of viable seeds would therefore not be able to reproduce under Norwegian growing condition.

There is no reason to assume that expression of the introduced characteristics in soybean 40-3-2 will increase the potential to establish feral populations. A series of field trials with soybean 40-3-2 was conducted by the applicant at several locations in the USA and Puerto Rica (1991-1994), Argentina (1993-1994), Canada (1993-1994), France (1994), and Italy (1994, 1996, and 1997) to compare the agronomic performance and field characteristics of soybean 40-3-2 with its comparators (see section 3.3). The agronomic and phenotypic field trial data did not show major changes in plant characteristics indicating altered fitness, persistence and invasiveness of soybean 40-3-2 plants compared to its conventional counterpart, except in the presence of glyphosate herbicides.

In addition to the data presented by the applicant, the VKM GMO Panel is not aware of any scientific reports indicative of increased establishment or spread of soybean 40-3-2, or changes to its survivability (including over-wintering), persistence or invasive capacity. Because the general characteristics of soybean 40-3-2 are unchanged, the herbicide tolerance is not likely to provide a selective advantage outside of cultivation in Norway. The VKM GMO Panel is of the opinion that the likelihood of unintended environmental effects based on establishment and survival of soybean 40-3-2 will not differ from that of conventional soybean varieties.

5.2 Potential for gene transfer

A prerequisite for any gene transfer is the availability of pathways for the transfer of genetic material, either through horisontal gene transfer of DNA, or vertical gene flow via pollen or seed dispersal. Transgenic DNA is also a component of a variety of food and feed products derived from soybean 40-3-2. This means that micro-organisms in the digestive tract in humans and animals (both domesticated animals and other animals feeding on fresh or decaying plant material from the transgenic soybean) may be exposed to transgenic DNA.

5.2.1 Plant to micro-organisms gene transfer

Experimental studies have shown that gene transfer from transgenic plants to bacteria rarely occurs under natural conditions and that such transfer depends on the presence of DNA sequence similarity between the DNA of the transgenic plant and the DNA of the bacterial recipient (Nielsen et al. 2000; De Vries & Wackernagel 2002, reviewed in EFSA 2004, 2009a; Bensasson et al. 2004; VKM 2005).

Based on established scientific knowledge of the barriers for gene transfer between unrelated species and the experimental research on horisontal transfer of genetic material from plants to microorganisms, there is today little evidence pointing to a likelihood of

random transfer of the transgene present in soybean 40-3-2 to unrelated species such as bacteria.

It is however pointed out that there are limitations in the methodology used in these experimental studies (Nielsen & Townsend 2004). Experimental studies of limited scale should be interpreted with caution given the scale differences between what can be experimental investigation and commercial plant cultivation.

Experiments have been performed to study the stability and uptake of DNA from the intestinal tract in mice after M13 DNA was administered orally. The DNA introduced was detected in stool samples up to seven hours after feeding. Small amounts (<0.1%) could be traced in the blood vessels for a period of maximum 24 hours, and M13 DNA was found in the liver and spleen for up to 24 hours (Schubbert et al. 1994). By oral intake of genetically modified soybean it has been shown that DNA is more stable in the intestine of persons with colostomy compared to a control group (Netherwood et al. 2004). No GM DNA was detected in the faeces from the control group. Rizzi et al. (2012) provides an extensive review of the fate of feed-derived DNA in the gastrointestinal system of mammals.

In conclusion, the VKM GMO Panel considers it is unlikely that the introduced gene from soybean 40-3-2 will transfer and establish in the genome of microorganisms in the environment or in the intestinal tract of humans or animals. In the rare, but theoretically possible case of transfer of the *cp4 epsps* gene from 40-3-2 to soil bacteria, no novel property would be introduced into or expressed in the soil microbial communities; as these genes are already present in other bacteria in soil. Therefore, no positive selective advantage that would not have been conferred by natural gene transfer between bacteria is expected.

5.2.2 Plant to plant gene flow

The genus *Glycine* has two distinct subgenera; *Glycine* and *Soya.* The subgenus *Glycine* contains 16 perennial wild species, whilst cultivated soybean (*G. max*) and its wild and semi-wild annual relatives, *G. soja* and *G. gracilis* are classified in the subgenus *Soja* (OECD 2000). Wild soybean species are endemic to China, Korea, Japan, Taiwan and the former USSR, and the species have not been reported in Europe or in North America.

Soybean is predominantly a self-pollinating species, propagated commercially by seed. The percentage of cross-pollinating is usually less than one percent (Lu 2005; OECD 2000). The dispersal of pollen is limited because the anthers mature in the bud and directly pollinate the stigma of the same flower. Pollination and fertilisation are usually accomplished before the flower opens (Acquaah 2012).

Since there is no cultivation of soybean in Norway and the species has no sexually compatible wild relatives in Europe, accidental seed spillage during transportation and/or

processing of soybean 40-3-2 will not present a risk of spread of transgenes to organic or conventionally grown varieties, or wild populations and closely related species in Norway.

5.3 Interactions between the GM plant and target organisms

Considering the intended uses of soybean 40-3-2, excluding cultivation and the absence of target organisms, potential interactions of the GM plant with target organisms were not considered an issue by the VKM GMO Panel.

5.4 Potential interactions between the GM plant and non-target organisms (NTOs)

Considering the intended uses of soybean 40-3-2, excluding cultivation, potential interactions of the GM maize with non-target organisms were not considered an issue by the VKM GMO Panel.

5.5 Potential interactions with the abiotic environment and biochemical cycles

Considering the intended uses of soybean 40-3-2, which exclude cultivation, and the low level of exposure to the environment, potential interactions of the GM plant with the abiotic environment and biogeochemical cycles were not considered an issue by the VKM GMO Panel.

5.6 Conclusion

Considering the intended uses of soybean 40-3-2, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure, mainly through manure and faeces from animals fed grains from soybean 40-3-2.

Soybean 40-3-2 has no altered survival, multiplication or dissemination characteristics compared to conventional soybean, and there are no indications of an increased likelihood of spread and establishment of feral soybean plants in the case of accidental release into the environment of seeds from soybean 40-3-2. Soybean is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of soybean in Europe. Plants to plant gene flow are therefore not considered to be an issue. Considering the intended use as food and feed, interactions with the biotic and abiotic environment are not considered to be an issue.

6 Post-market environmental monitoring

Directive 2001/18/EC introduces an obligation for applicants to implement monitoring plans, in order to trace and identify any direct or indirect, immediate, delayed or unanticipated effects on human health or the environment of GMOs as or in products after they have been placed on the market. Monitoring plans should be designed according to Annex VII of the Directive. According to Annex VII, the objectives of an environmental monitoring plan are 1) to confirm that any assumption regarding the occurrence and impact of potential adverse effects of the GMO or its use in the environmental risk assessment (ERA) are correct, and (2) to identify the occurrence of adverse effects of the GMO or its use on human health or the environment which were not anticipated in the environmental risk assessment.

Post-market environmental monitoring is composed of case-specific monitoring and general surveillance (EFSA 2011c). Case-specific monitoring is not obligatory, but may be required to verify assumptions and conclusions of the ERA, whereas general surveillance is mandatory, in order to take account for general or unspecific scientific uncertainty and any unanticipated adverse effects associated with the release and management of a GM plant. Due to different objectives between case-specific monitoring and general surveillance, their underlying concepts differ. Case-specific monitoring should enable the determination of whether and to what extent adverse effects anticipated in the environmental risk assessment occur during the commercial use of a GM plant, and thus to relate observed changes to specific risks. It is triggered by scientific uncertainty that was identified in the ERA.

The objective of general surveillance is to identify unanticipated adverse effects of the GM plant or its use on human health and the environment that were not predicted or specifically identified during the ERA. In contrast to case-specific monitoring, the general status of the environment that is associated with the use of the GM plant is monitored without any preconceived hypothesis, in order to detect any possible effects that were not anticipated in the ERA, or that are long-term or cumulative.

No specific environmental impact of genetically modified soybean 40-3-2 was indicated by the environmental risk assessment and thus no case specific monitoring is required. The VKM GMO Panel is of the opinion that the monitoring plan provided by the applicant is in line with the intended uses of soybean 40-3-2.

7 Conclusions

Molecular characterisation

The applicant has provided sufficient analyses to characterise the DNA inserts, number of inserts, integration sites and flanking sequences in the soybean genome. The results show that one functional copy of the *cp4 epsps* gene only, is present in the soybean 40-3-2 genome. No other functional vector genes were found. Updated similarity searches in 2010, with databases of known toxins and allergens did not indicate a potential production of harmful proteins or polypeptides caused by the genetic modification. Southern blot analyses and segregation studies show that the introduced gene is stably inherited and expressed over multiple generations along with the phenotypic characteristics of soybean 40-3-2. The VKM GMO Panel concludes that the molecular characterisation of soybean 40-3-2 does not indicate a safety concern.

Comparative assessment

The VKM GMO Panel has considered the available literature on compositional data and found no biologically meaningful differences between soybean 40-3-2 and the conventional non-GM control, except small intermittent variations. The data presented do not show unintended effects as a result of the genetic modification. The VKM GMO Panel concluded that soybean 40-3-2 is compositional, agronomical and phenotypically equivalent to its conventional counterpart, and other conventional soybean varieties.

Food and feed risk assessment

Subchronic feeding studies on the glyphosate-tolerant soybean 40-3-2 in rats, as well as whole food feeding studies on broilers, quails, cows, pigs, piglets, catfish and Atlantic salmon have not indicated any adverse effects. The CP4 EPSPS protein does not show sequence resemblance to known toxins or IgE allergens, nor has it been reported to cause IgE mediated allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that soybean 40-3-2 is nutritionally equivalent and as safe as conventional soybean.

Environmental assessment

Considering the intended uses of soybean 40-3-2, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure, mainly through manure and faeces from animals fed grains from soybean 40-3-2.

Soybean 40-3-2 has no altered survival, multiplication or dissemination characteristics compared to conventional soybean, and there are no indications of an increased likelihood of spread and establishment of feral soybean plants in the case of accidental release into the environment of seeds from soybean 40-3-2. Soybean is not cultivated in Norway, and there are no cross-compatible wild or weedy relatives of soybean in Europe. Plants to plant gene flow are therefore not considered to be an issue. Considering the intended use as food and feed, interactions with the biotic and abiotic environment are not considered to be an issue.

Overall conclusion

Based on current knowledge, the VKM GMO Panel concludes that soybean 40-3-2 is as safe as its conventional counterpart and commercial soybean varieties with the intended usage. Soybean 40-3-2 is nutritionally, phenotypically and agronomically equivalent to conventional soybean varieties.

Likewise, the VKM GMO Panel concludes that soybean 40-3-2, based on current knowledge, does not represent an environmental risk in Norway with the intended usage.

8 Data gaps

Herbicide tolerant (HT) crops permit the use of broad-spectrum herbicides such as glyphosate, as an in-crop selective herbicide to control a wide range of broadleaf and grass weeds without sustaining crop injury. This weed management strategy enables post-emergence spraying to established weeds and gives growers more flexibility to choose spraying times in comparison with the pre-emergence treatments of conventional crops.

As the broad-spectrum herbicides are sprayed on the plant canopy and often takes place later in the growing season than is the case with selective herbicides associated with conventional crops, the residue and metabolite levels of herbicides in plants with tolerance to glyphosate could be higher compared to plants produced by conventional farming practices. There are however limited amounts of data available on herbicide residues in HT crops.

More research is needed to elucidate whether the genetic modifications used to make a plant tolerant against certain herbicide(s) may influence the metabolism of this or other plant protection products, and whether possible changes in the spectrum of metabolites may result in altered toxicological properties.

At present, the potential changes related to herbicide residues of genetically modified plants as a result of the application of plant protection products fall outside the remit of the Norwegian Scientific Committee for Food Safety.

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Appendix I

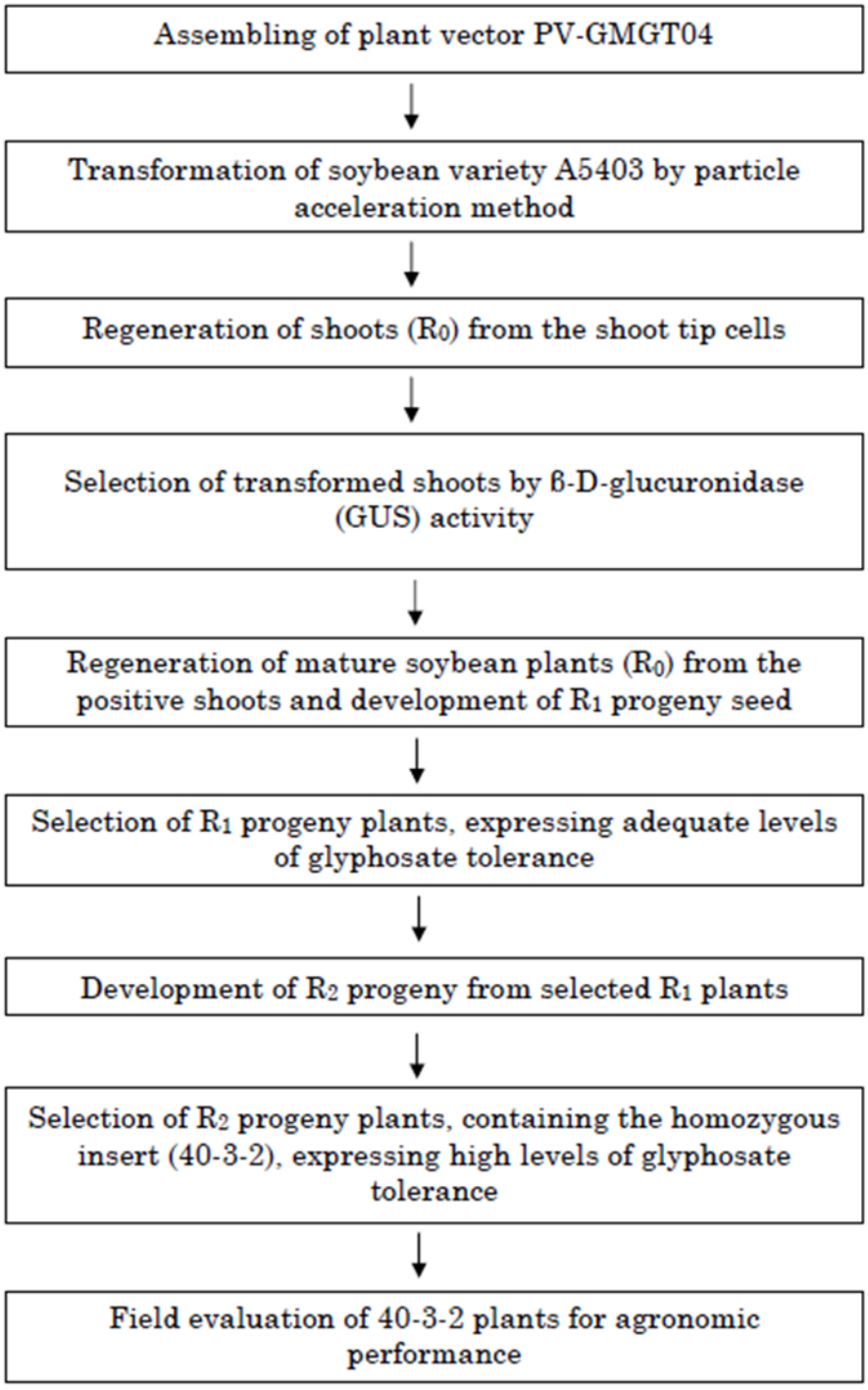

Figure AI-1. Flow chart for the development of soybean 40-3-2 (figure 2 in Technical dossier).

Table AI-1. Updated bioinformatics analyses (From Monsanto technical report Tu and Silvanovich (2010b)

Application EFSA-GMO-RX-40-3-2 (Art. 8.1b and 20.1b)

Responses to EFSA questions
(Ref. PB/KL/ZD/shv (2010)4722621)

13 July 2010

Table 1 Flanking sequences (disruption of known coding sequences or regulatory elements)

	General Database *	Date**	Algorithms ***	Reference ****	EST Database *	Date**	Algorithms ***	Reference ****
40-3-2	NT_2010 (GenBank non-redundant nucleotides)	22 January 2010	BLASTn	Tu and Silvanovich (2010b) attached to this response document	EST_2010 (GenBank EST database)	22 January 2010	BLASTn	Tu and Silvanovich (2010b) attached to this response document
	NR_2010 (GenBank non-redundant amino acids)	22 January 2010	BLASTx	Tu and Silvanovich (2010b) attached to this response document	NA	NA	NA	NA

* Name of the database e.g. Genbank non-redundant nucleotide, Genbank non-redundant protein, Genbank general/plant/species EST, FARRP (incl version, using official name)
** Date of last update of the database
*** Algorithm e.g. BLASTn, BLASTx, BLASTp, Fasta,…
**** Application number; place in dossier (e.g. technical dossier, additional information); reference name.
NA: Not Applicable

Table AI-2. Updated bioinformatics analyses (From Monsanto technical report Tu and Silvanovich (2010b)

Application EFSA-GMO-RX-40-3-2 (Art. 8.1b and 20.1b)

Responses to EFSA questions
(Ref. PB/KL/ZD/shv (2010)4722621) 13 July 2010

Table 2 Junction regions - ORF analyses (potential creation of new ORFs having similarity to known allergens or toxins)

	Allergen database *	Date**	Algorithms ***	Reference ****	Toxin-database *	Date**	Algorithms ***	Reference ****	General database *	Date**	Algorithms ***	Reference ****
40-3-2	AD_2010	22 January 2010	FASTA and 8 amino acid sliding window	Tu and Silvanovich (2010b) attached to this response document	TOX_2010	22 January 2010	FASTA	Tu and Silvanovich (2010b) attached to this response document	PRT_2010 (GenBank 175)	22 January 2010	FASTA	Tu and Silvanovich (2010b) attached to this response document

* Name of the database e.g. Genbank non-redundant nucleotide, Genbank non-redundant protein, Genbank general/plant/species EST, FARRP (incl version, using official name)
** Date of last update of the database
*** Algorithm e.g. BLASTn, BLASTx, BLASTp, Fasta,…
**** Application number; place in dossier (e.g. technical dossier, additional information); reference name.
NA: Not Applicable.

Table AI-3. Updated bioinformatics analyses (From Monsanto technical report Tu and Silvanovich (2010b)

Application EFSA-GMO-RX-40-3-2 (Art. 8.1b and 20.1b)

Responses to EFSA questions 13 July 2010
(Ref. PB/KL/ZD/shv (2010)4722621)

Table 3 Newly expressed proteins

	Allergen database *	Date**	Algorithms ***	Reference ****	Toxin-database *	Date**	Algorithms ***	Reference ****
CP4 EPSPS	AD_2010	22 January 2010	FASTA and 8 amino acid sliding window	Tu and Silvanovich (2010b) attached to this response document	TOX_2010	22 January 2010	FASTA	Tu and Silvanovich (2010b) attached to this response document

* Name of the database e.g. Genbank non-redundant nucleotide, Genbank non-redundant protein, Genbank general/plant/species EST, FARRP (incl version, using official name)
** Date of last update of the database
*** Algorithm e.g. BLASTn, BLASTx, BLASTp, Fasta,…
**** Application number; place in dossier (e.g. technical dossier, additional information); reference name.
NA: Not Applicable

Table AI-4. Segregation data for F_2 progeny of crosses between traditional soybean varieties and soybean 40-3-2 (Table 6 in Technical dossier)

Family	Tolerant	Sensitive	X^2 *
1	17	4	0.4
2	10	2	0.44
3	12	4	0
4	16	4	0.27
5	16	5	0.02
6	14	3	0.49
7	18	5	0.13
8	10	4	0.1
9	17	7	0.22
10	6	3	0.33
11	15	4	0.16
12	17	1	3.63
13	10	1	1.48
14	16	5	0.02
15	3	1	0
16	18	3	1.29
17	19	5	0.22
Total	234	61	2.94

* Uncorrected Chi-square goodness-of-fit test for hypothesis of 3:1 segregation. None of the Chi-square values are significant at the 95% confidence level ($X^2_{0.05,1\ d.f.}$=3.84).

Table AI-5. Supplemental segregation data on soybean 40-3-2 (Table 7 in Technical dossier)

Family	Null[a]	Heterozygous[a]	Homozygous[a]	X^2 [b]
DBL3201AOX	4	15	10	2.47
DKB2301A1R	3	19	8	3.8

[a] Zygosity determined by PCR-based assay.
[b] Uncorrected Chi-square goodness-of-fit test for hypothesis of 1:2:1 segregation. None of the Chi-square values are significant at the 95% confidence level

Table AI-6. Proximate and Fiber composition in Forage from Glyphosate-tolerant Soybean 40-3-2 (Harrigan et al., 2007)

component[a]	40-3-2 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	lit.[f] (range)[d]	ILSI[g] (range)[d]
moisture	70.56 (66.30–75.50)	70.50 (64.50–75.90)	(51.70–75.00) [49.14, 85.29]	74–79	73.5–81.6
protein	18.84 (14.74–31.37)	18.50 (15.88–23.65)	(12.72–22.73) [14.16, 23.63]	11.2–17.3	14.38–24.71
fat	5.39 (1.98–6.91)	5.04 (2.68–6.64)	(2.91–8.67) [2.30, 9.95]	3.1–5.1	1.30–5.13
ash	6.64 (5.34–7.56)	6.71 (5.72–8.26)	(4.68–9.24) [3.61, 9.34]	8.8–10.5	6.72–10.78
carbohydrates	69.13 (60.09–75.63)	69.74 (64.89–73.09)	(62.95–74.67) [60.96, 76.06]	na[i]	59.8–74.7
ADF[h]	31.93[j] (26.38–35.71)	30.26 (26.88–33.82)	(22.72–37.92) [22.52, 39.00]	32–38	na[i]
NDF[h]	38.62 (31.16–64.89)	34.94 (29.44–39.26)	(27.65–52.22) [20.61, 52.66]	34–40	na[i]

[a] Percent dry weight of sample, except moisture. [b] Nontransgenic control. [c] Commercial nontransgenic varieties planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial soybean population where negative limits are set to zero. [f] OECD, 2001 (*44*). [g] International Life Sciences Institute crop composition database, Ridley et al. (*45*). [h] ADF, acid detergent fiber; NDF, neutral detergent fiber. [i] na, not available. [j] Statistically different from the control at the 5% level ($p < 0.05$).

Table AI-7. Proximate and fiber composition of harvested seed from Glyphosate-Tolerant Soybean 40-3-2 (Harrigan et al., 2007)

component[a]	40-3-2 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	lit. (range)[d]	ILSI[j] (range)[d]
moisture	5.38 (4.90–5.81)	5.44 (4.97–5.71)	(4.71–5.76) [4.64, 6.18]	5.3–8.73[f], 5.18–14.3[g]	4.7–34.4
protein	37.73 (32.86–40.83)	38.48 (33.41–41.24)	(32.54–42.50) [31.19, 45.73]	329–436[h] g/kg of dw 360–484[f] g/kg of dw	33.19–45.48
fat	17.28 (15.79–19.04)	17.39 (15.80–20.10)	(15.16–20.28) [14.29, 21.82]	198–267[h] g/kg of dw 160–231[f] g/kg of dw	8.10–23.56
ash	5.49 (4.86–6.27)	5.54 (5.06–6.44)	(4.53–6.23) [4.59, 6.15]	4.61–5.94[g]; 4.29–5.88[f]	3.88–6.99
carbohydrates	39.50 (34.98–45.11)	38.58 (35.56–44.35)	(33.84–42.39) [31.67, 44.56]	29.3–41.3[f]	29.6–50.2
ADF[k]	16.85 (13.29–19.19)	16.94 (13.78–19.36)	(11.85–21.84) [10.78, 22.71]	na[l]	7.81–18.61
NDF[k]	18.57 (13.40–22.14)	18.54 (13.46–22.96)	(12.77–23.30) [9.55, 25.96]	na[l]	8.53–21.25
crude fiber	12.87 (9.74–14.43)	12.76 (9.82–14.29)	(7.32–16.33) [8.39, 16.70]	na[l]	4.12–13.87

[a] Percent dry weight of sample, except moisture. [b] Nontransgenic control. [c] Commercial nontransgenic varieties planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial soybean population where negative limits are set to zero. [f] Padgette et al. (*6*). [g] Taylor et al. (*7*). [h] Maestri et al. (*46*). [i] Hartwig and Kilen (*47*). [j] International Life Sciences Institute crop composition database, Ridley et al. (*45*). [k] ADF, acid detergent fiber; NDF, neutral detergent fiber. [l] na, not available.

Table AI-8. Amino acid composition of harvested seed from Glyphosate-tolerant Soybean 40-3-2 (Harrigan et al., 2007)

component[a]	40-3-2 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	lit.[f] (range)[d]	ILSI[a,g] (range)[d]
alanine	1.71 (1.56–1.82)	1.73 (1.61–1.81)	(1.56–1.88) [1.51, 1.96]	1.60–1.86	15.13–21.04
arginine	2.76 (2.46–3.06)	2.83 (2.55–3.15)	(2.42–3.36) [2.20, 3.60]	2.56–3.46	22.85–34.00
aspartic acid	4.35 (3.98–4.76)	4.41 (4.07–4.77)	(3.90–4.82) [3.75, 5.15]	4.18–4.99	38.08–51.22
cystine	0.60 (0.57–0.67)	0.59 (0.55–0.66)	(0.50–0.66) [0.46, 0.70]	0.54–0.66	3.70–8.08
glutamic acid	6.84 (6.10–7.43)	6.95 (6.33–7.52)	(5.97–7.87) [5.66, 8.43]	6.64–8.16	58.43–82.01
glycine	1.64 (1.50–1.79)	1.65 (1.52–1.78)	(1.45–1.81) [1.41, 1.93]	1.60–1.87	14.58–19.97
histidine	1.01 (0.93–1.10)	1.02 (0.95–1.10)	(0.92–1.12) [0.89, 1.17]	0.98–1.16	8.78–11.75
isoleucine	1.69[h] (1.54–1.85)	1.73 (1.56–1.85)	(1.51–1.89) [1.43, 2.04]	1.65–1.95	15.39–20.77
leucine	2.93 (2.70–3.16)	2.98 (2.76–3.21)	(2.62–3.27) [2.55, 3.44]	2.81–3.37	25.90–36.22
lysine	2.46 (2.31–2.67)	2.48 (2.33–2.64)	(2.26–2.69) [2.19, 2.83]	2.47–2.84	22.85–28.39
methionine	0.56 (0.49–0.62)	0.55 (0.50–0.61)	(0.49–0.61) [0.46, 0.64]	0.51–0.59	4.31–6.81
phenylalanine	1.95 (1.73–2.07)	1.99 (1.84–2.11)	(1.75–2.23) [1.68, 2.35]	1.78–2.19	16.32–23.46
proline	1.91 (1.72–2.07)	1.95 (1.80–2.10)	(1.70–2.19) [1.63, 2.29]	1.86–2.23	16.87–22.84
serine	2.03 (1.86–2.17)	2.04 (1.92–2.19)	(1.83–2.25) [1.80, 2.34]	1.96–2.28	11.06–24.84
threonine	1.54 (1.45–1.68)	1.54 (1.46–1.68)	(1.41–1.67) [1.37, 1.71]	1.51–1.73	11.39–18.62
tryptophan	0.45 (0.34–0.52)	0.47 (0.41–0.53)	(0.36–0.55) [0.33, 0.58]	0.56–0.63	3.56–5.02
tyrosine	1.31 (1.19–1.43)	1.33 (1.24–1.44)	(1.12–1.48) [1.14, 1.54]	1.35–1.59	10.16–16.13
valine	1.80[h] (1.65–1.96)	1.84 (1.67–1.96)	(1.61–1.99) [1.53, 2.16]	1.71–2.02	15.97–22.04

[a] Percent dry weight of sample except for ILSI column, where data are reported as mg/g dry weight; conversion formula % DW = [mg/g] × 0.1. [b] Nontransgenic control. [c] Commercial nontransgenic varieties planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial soybean population where negative limits set to zero. [f] Padgette et al. (*8*). [g] International Life Sciences Institute crop composition catabase, Ridley et al. (*45*). [h] Statistically different from the control at the 5% level ($p < 0.05$).

Table AI-9. Fatty acid composition of Harvested seed from Glyphosate-tolerant Soybean 40-3-2 (Harrigan et al., 2007)

fatty acid[a]	40-3-2 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	lit.[f,g] (range)[d]	ILSI[h,i] (range)[d]
16:0 palmitic	1.79 (1.60–2.01)	1.82 (1.62–2.06)	(1.40–2.16) [1.08, 2.49]		
	10.80 (10.54–11.06)	*10.93 (10.65–11.15)*	*(9.16–12.07)*	*10.63–11.69*	*9.55–15.77*
18:0 stearic	0.84 (0.58–1.11)	0.84 (0.60–1.10)	(0.50–1.16) [0.33, 1.31]		
	5.05 (3.64–6.37)	*5.06 (3.78–6.13)*	*(3.30–6.58)*	*3.85–4.55*	*2.70–5.88*
18:1 oleic	3.51 (2.80–4.20)	3.49 (2.80–4.27)	(2.60–4.75) [1.68, 5.62]		
	21.10 (17.39–23.32)	*20.91 (17.51–23.11)*	*(15.80–25.93)*	*15.02–31.19*	*14.3–32.2*
18:2 linoleic	8.80 (7.91–9.91)	8.80 (8.04–9.79)	(7.58–10.59) [6.77, 11.85]		
	53.10 (50.67–56.90)	*52.99 (50.72–56.60)*	*(48.40–59.49)*	*44.03–54.96*	*48.2–58.8*
18:3 linolenic	1.49 (1.31–1.81)	1.52 (1.32–1.76)	(1.27–1.98) [0.93, 2.19]		
	9.04 (7.97–10.42)	*9.19 (8.02–10.69)*	*(7.47–11.24)*	*5.08–10.26*	*3.00–12.52*
20:0 arachidic	0.063 (0.045–0.083)	0.063 (0.046–0.080)	(0.038–0.080) [0.029, 0.093]		
	0.37 (0.28–0.46)	*0.38 (0.29–0.45)*	*(0.25–0.47)*	*0.31–0.43*	*0.16–0.48*
20:1 eicosenoic	0.030 (0.025–0.037)	0.031 (0.026–0.039)	(0.024–0.045) [0.015, 0.053]		
	0.18 (0.16–0.21)	*0.19 (0.16–0.21)*	*(0.16–0.24)*	*0.14–0.26*	*0.14–0.35*
22:0 behenic	0.058 (0.047–0.072)	0.059 (0.049–0.069)	(0.043–0.072) [0.040, 0.078]		
	0.35 (0.30–0.39)	*0.36 (0.31–0.40)*	*(0.28–0.42)*	*0.46–0.59*	*0.28–0.59*

[a] Values in italics are percentage dry weight; values of fatty acids expressed as percent of total fatty acid are presented in regular font. Statistical analyses were performed on data expressed as percent dry weight, whereas the percent total fatty acid data are presented to facilitate comparisons with literature and ILSI data. The analytical method included the measurement of the following fatty acids that were not detected in the majority of samples analyzed: caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), myristic acid (14:0), myristoleic acid (14:1), pentadecanoic acid (15:0), pentadecenoic acid (15:1), palmitoleic acid (16:1), heptadecanoic acid (17:0), heptadecenoic acid (17:1), γ-linolenic (18:3), eicosadienoic acid (20:2), eicosatrienoic acid (20:3), and arachidonic acid (20:4). [b] Nontransgenic control. [c] Commercial nontransgenic varieties planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial soybean population where negative limits set to zero. [f] Padgette et al. (*6*). [g] Values expressed as percent of total fat except for palmitic acid (16:1) that is expressed as percent of triglyceride fatty acids. [h] International Life Sciences Institute crop composition database, Ridley et al. (*45*). [i] Values expressed as percent of total fat.

Table AI-10. Isoflavone Composition from Harvested seed from Glyphosate Tolerant Soybean 40-3-2 (Harrigan et al, 2007)

isoflavone[a]	40-3-2 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] (99% tolerance interval)[e]	lit.[f] (range)[d]	ILSI[g] (range)[d]
daidzein	1161.82 (755.26–1663.86)	1171.77 (713.00–1702.08)	(567.39–2094.57) [0, 2822.32]	9.88–124.2	60.0–2453.5
genistein	1641.80[h] (988.70–2379.95)	1717.00 (911.88–2600.70)	(613.79–2367.61) [0, 3354.84]	13–150.1	144.3–2837.2
glycitein	86.80 (46.00–151.26)	90.55 (65.95–132.21)	(46.16–349.19) [0, 281.87]	4.22–20.4	15.3–310.4

[a] Units expressed as μg/g of dry weight. [b] Nontransgenic control. [c] Commercial nontransgenic varieties planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial soybean population where negative limits are set to zero. [f] USDA-ISU Isoflavone Database (48) with units expressed as mg/100 g of fw. [g] International Life Sciences Institute crop composition database, Ridley et al. (45). [h] Statistically different from the control at the 5% level ($p < 0.05$).

Table AI-11. Composition of antinutrients of Harvested seed from Glyphosate Tolerant Soybean 40-3-2 (Harrigan et al, 2007)

component[a]	40-3-2 mean (range)[d]	control[b] mean (range)[d]	commercial references[c] (range)[d] [99% tolerance interval][e]	lit.[f,g] (range)[d]	ILSI[h] (range)[d]
raffinose	0.33 (0.24–0.43)	0.35 (0.25–0.46)	(0.22–0.63) [0.032, 0.75]		0.21–0.66
stachyose	2.25 (1.43–2.81)	2.43 (1.76–3.37)	(1.52–3.28) [1.07, 3.64]		1.21–3.50
trypsin inhibitor (TIU/mg of DW)	32.10 (23.64–43.45)	32.63 (25.77–57.77)	(21.41–66.00) [1.00, 62.87]	33.2–54.5[f]	19.59–118.68
lectin (HU/mg of FW)	1.20 (0.62–2.39)	1.40 (0.68–1.99)	(0.26–4.53) [0, 3.29]	0.8–2.4[f] 37–323[g] HU/mg of protein	0.09–8.46
phytic acid	1.17 (0.77–1.78)	1.13 (0.67–1.53)	(0.56–1.93) [0.16, 2.09]		0.634–1.960

[a] Units expressed as percent dry weight except trypsin inhibitos and lectins that are expressed as noted. [b] Nontransgenic control. [c] Commercial nontransgenic varieties planted at each site. [d] Range denotes the lowest and highest individual values across all sites. [e] Tolerance interval is specified to contain 99% of the commercial soybean population where negative limits are set to zero. [f] Padgette et al. (6). [g] Kakade et al. (49). [h] International Life Sciences Institute crop composition database, Ridley et al. (45).

Appendix II

Soy products

By Dagrunn Engeset and Inger Therese Lillegaard

There are different soy-products on the market: milk replacement products (milk, sour cream, yoghurt, and cheeses), meat replacement products (soy granules to mix in water to make “minced meat “, and ready made products like sausages, burgers, nuggets, and schnitzels), desserts (vanilla and chocolate puddings, ice creams, cheese cakes), soy flour, soy flakes, soy beans, soy fat/oils, and –sauce. There are also soy proteins in several diet bars and diet products, and in a few canned meat products. Many chocolates and biscuits contain soy lecithin.

In this project two different menus have been created; one full day week menu for a person with milk allergy and one full day week menu for a vegan (see below). We wanted to examine how much soy protein a person can get, realistically, by replacing meat and milk products with soy-products.

Reason for the choice of menus

The milk allergy menu
Milk allergy or intolerance is relatively common diseases. Persons with such diseases will have to look for alternatives to milk and milk products, and soy products will be a natural choice for many of them. There are other milk replacement products on the market, but in this scenario we envision a person who prefers soy over other products. This menu is also relevant for persons who for various reasons do not want to use milk products and therefore replaces them with soy products.

The vegan menu
A vegan does not eat any products of animal origin; meat, fish, milk, and egg. In this scenario we envision a vegan who has previously eaten normal food and wish to replace meat products with meat replacement products like soy sausages and-burgers in addition to replacing milk products. In both menus all milk products are replaced with soy products: soy milk substitute milk for drinking, milk in waffles, milk in porridge and on breakfast cereals, in smoothies, and in cheese sauces.

Coffee milk is substituted with soy cream in coffee or tea.
Cheeses are replaced by different soy cheeses and/or tofu on bread, and in dishes like lasagne and pizza. Tofu is also used in cheese cake, smoothies, and in salads.

Soy yoghurt, ice cream, cream, and sour cream replace ordinary yoghurt, ice cream, cream, and sour cream. In the vegan menu meat products are replaced by meat substitutes of soy and of tofu in wraps and in lasagne.
The menus are made with an estimated energy requirement of 10MJ/day. We assume that in pure soy products (e.g. soy milk) all the protein come from soy. In mixed products the amount of soy protein is estimated based on how much soy was stated in the table of content printed on the food label.

7 days vegan menu, high preference for soy products
(envision a person who has previously eaten meat and is looking for meat substitutes like soy burgers and sausages)

Monday:
Breakfast: Cereals with nuts and soy milk, orange juice, coffee/tea with soy cream
Lunch: course bread with soy cheese, cucumber and tomato, bell pepper, peanut butter, soy milk, coffee/tea with soy cream
Snack: banana, walnuts
Dinner: soy burger, burger bread, tomato, lettuce, pickles, raw onion, soy cheese, soy chocolate dessert, water
Supper: mixed salad with tofu, vinaigrette dressing and pita bread, tea

Tuesday:
Breakfast: cereals with nuts and soy milk, orange juice, coffee with soy cream (like Monday)
Lunch: tofu wrap (tortilla with tofu + vegetables), soy milk, coffee with soy cream
Snack: apple, soy ice cream
Dinner: Steamed vegetables with cheese sauce (made of soy milk and soy cheese), water, soy yoghurt with nuts and raisins
Supper: oat porridge with raisins and soy milk

Wednesday:
Breakfast: Soy smoothie (tofu, soy milk, banana, strawberries)
Lunch: tofu wrap, soy milk, coffee (like Tuesday)
Snack: soy yoghurt
Dinner: Soy sausages , mixed salad with tofu, rice, water, vanilla soy dessert
Supper: course bread with peanut butter, soy cheese and vegetables, soy milk and coffee (like lunch Monday)

Thursday:
Breakfast: cereals with nuts and soy milk, orange juice, coffee with soy milk
Lunch: bread lunch like Monday
Snack: Soy smoothie (like breakfast Wednesday)
Dinner: Vegetable soup, course rye bread with milk free margarine, water

Supper: bread with peanut butter, soy cheese, bell pepper, coffee with soy cream, orange juice

Friday:
Breakfast: bread breakfast (like Thursday supper)
Lunch: mixed salad with tofu (like Monday supper)
Snack: Soy waffle with jam and soy sour cream (waffles of soy milk, peanut butter, soy oil, buck wheat, corn starch, corn flour), soy chocolate milk (hot) with whipped cream (soy whipping spray cream)
Dinner: Spinach and tofu lasagne (lasagne plates, spinach, tofu, soy milk, soy cheese, tomato sauce) with mixed salad and white bread, wine and water
Supper: fruit salad

Saturday:
Breakfast: Soy smoothie (as previous)
Lunch: Soy waffle (like Friday snack)
Snack: Milk chocolate without milk, cashew nuts, raspberries
Dinner: Vegetarian bean casserole, pita bread, wine, water, soy chocolate dessert
Supper: Vegan pizza (marguerita with soy cheese), beer, potato chips

Sunday:
Breakfast: soy sausages, chapatti, onion, pickles, tomato juice, tea
Lunch: tofu wrap (like lunch Tuesday)
Snack: fruit salad
Dinner: Vegan meatballs (chickpeas, tofu, water, rolled oats, wheat flour) in tomato sauce, spaghetti, mixed salad, soda, soy chocolate dessert
Supper: vegan cheesecake with raspberries (cheese cream topping: soy cream cheese, tofu, sugar, lemon), coffee

7 day menu, milk allergy - replaces milk products with soy products.

Monday:
Breakfast: Oat porridge (like vegan)
Lunch: Bread with salami and soy cheese, tomato/cucumber/bell pepper, orange juice, coffee
Snack: Banana, walnuts
Dinner: Sausages without milk, mashed potatoes with soy milk, mixed salad, water
Supper: Coarse bread, boiled egg, pickled herring, milk free margarine, mayonnaise, soy milk

Tuesday:
Breakfast: Bread breakfast (like Monday lunch)

Lunch: Bread lunch (like Monday supper)
Snack: Smoothie (like vegan)
Dinner: Vegetable soup (like vegan Thursday)
Supper: omelette with bread, soy milk, tea

Wednesday:
Breakfast: Weetabix with soy milk
Lunch: Bread lunch (like Monday supper)
Snack: Banana and nuts
Dinner: Meat balls, mushy peas, potatoes, carrots, sauce, lingonberry jam, water
Supper: Oat porridge (like vegan)

Thursday:
Breakfast: Smoothie (soy milk, strawberries, banana, apple juice)
Lunch: Bread lunch (like Monday supper)
Snack: Soy yoghurt with nuts, grapes
Dinner: Fish gratin made with soy milk, carrots, bacon, water, soy chocolate dessert
Supper: oat porridge (like vegan)

Friday:
Breakfast: Corn flakes with soy milk, coffee, orange juice
Lunch: Tomato soup with macaroni (without milk), white bread, water
Snack: Milk chocolate without milk, cashew nuts, raspberries
Dinner: Lasagne (cheese sauce of soy milk and soy cheese), mixed salad, pita bread, wine, water, soy ice cream
Supper: Pizza with soy cheese, beer, potato chips

Saturday:
Breakfast: Egg and bacon, bread, orange juice, coffee
Lunch: Mixed salad with chicken and tofu, pita bread, water
Snack: Smoothie (like Thursday breakfast)
Dinner: Rice porridge made with soy milk, mutton ham, lemonade
Supper: Taco with soy sour cream and soy cheese, beer

Sunday:
Breakfast: Omelette with soy cheese, bread, cucumber/bell pepper, orange juice, tea
Lunch: waffle with soy milk (ordinary waffle with egg where soy milk replaces milk) , jam, soy sour cream, coffee with soy cream and sugar
Snack: Milk free milk chocolate, nuts, fruit
Dinner: Salmon with potato, soy sour cream, cucumber, carrots, water, fruit salad
Supper: Vegan cheesecake with raspberries, coffee

Appendix III

A rather extensive safety testing programme has been conducted on soybean 40-3-2 within the Russian Federation and summarised in "Tutelyan VA (2013) Genetically Modified Food Sources. Safety Assessment and Control. Amsterdam: Academic Press, Elsevier. DOI: 10.1016/B978-0-12-405878-1.00009-4". The research and testing is claimed compliant with national requirements (MY 2.3.2.2306-07 "Medico-biological safety assessment of genetically-engineered and modified organisms of plant origin"). The content of these requirements and the exact design of the respective studies have been difficult to assess for the VKM GMO panel. A brief summary of the testing is thus presented as follows:

Study on in vivo genotoxicity

The potential genotoxicity of Soybean 40-3-2 was investigated in an *in vivo* genotoxicity experiment in mice (Tutelyan, 2013). The genotoxicity studies were carried out on C57Bl/6 and CBA mice sensitive to mutagenesis. For 35 days, the mice weighing 16–18 g were fed diet with protein concentrate from soybean 40-3-2 (test group) or its conventional counterpart (control group) with daily feed intake of 1.5 g/day/animal. These studies examined chromosomal aberrations in the cells of bone marrow and the dominant lethal mutations in the gametes of control and test mice. The cytogenetic analysis was carried out by metaphasic method (MUK 2.3.2.970-00, 2000). The mice of both groups were sacrificed 24 h after the last feeding. Two hours prior to termination of the experiment, the mice were intraperitoneally injected with colchicine to accumulate the cells with metaphases. Bone marrow was isolated from both femoral bones. A total of 70–80 cells at the metaphasic stage of nuclear division were taken for analysis from each mouse from the group of 2-month male C57Bl/6 mice weighing 20–22 g. Genetic alterations in gametes were examined by assessing dominant lethal mutations in C57Bl/6 male mice.

After the 35-day feeding period, the test and control male mice were caged with virgin CBA female mice in a ratio of 1:2. The mating period of 3 weeks was sufficient to assess the effect of soybean diet on sex cells (spermatids and spermatozoa) during the postmeiotic period. Pregnant females were isolated and sacrificed on gestation days 15–17 by cervical dislocation. Numbers of corpus lutea and live and dead embryos were recorded. These data were used to calculate the mutagenic parameters: pre-implantation, post-implantation, and inducible mortality.

The study examined chromosomal aberrations in the cells of bone marrow and the dominant lethal mutations in the gametes of control and test mice. Among various structural chromosomal abnormalities in animals of both groups, there were single segments, one circular chromosome (in a test mouse), and gaps. The number of cells with such chromosomal abnormalities did not significantly differ between control and test mice.

To examine the dominant lethal mutations, 90 test and 78 control females were dissected to analyse 473 (test) and 447(control) embryos, 502 test and 475 control implantation sites, and 537 (test) and 524 (control) corpus lutea. At the stages of early and late spermatids or mature spermatozoa, the pre-implantation mortality in the test group was lower than in the control. At these stages, the post-implantation mortality in the test group (the most significant index of mutagenic activity of the examined agent) did not surpass that in the control group. Induced mortality at the stages of early and late spermatids or mature spermatozoa was absent, indicating absence of the negative effect of the protein concentrate derived from transgenic soybean line 40-3-2 on spermiogenesis in mice. The investigators concluded that glyphosate-tolerant soybean line 40-3-2 produced no mutagenic effect in the described experiments.

Studies on immunotoxicity

The immunomodulating effect of GM soybean on the humoral component of the immune system was examined by determining the level of hemagglutination in mice after injecting sheep erythrocytes (SE) to mouselines C57Bl/6 (low sensitivity to SE) and CBA (high sensitivity to SE). Soybean protein concentrate was fed to mice for 21 days. The control and test mice were fed a diet with conventional and transgenic soybean line 40-3-2, correspondingly. On Day 21 the mice of both groups were intraperitoneally injected with 0.5 mL sheep erythrocytes (SE) (10 million cells). Blood was drawn on day 7, 14, and 21 after the onset of the experiment. Blood serum was titrated in reaction of hemagglutination by the routine method. All mice demonstrated the presence of antibodies against SE. At any term of the experiment, the antibody titers were 1:64 in C57Bl/6 mice and 1:128 in CBA mice. The control and test mice had identical titers of antibodies raised against SE. The investigators concluded that soybean line 40-3-2 produces no effect on the humoral component of the immune system compared to control.

The possible immunomodulating effect of transgenic soybean was further assessed with delayed hypersensitivity reaction to sheep erythrocytes (SE). The same mouse strain (C57Bl/6 - and CBA) and the same feeding regime as described above, was used in this test. The soybean protein concentrate was added to the diet for 21 days; thereafter, sheep erythrocytes (SE) was injected subcutaneously (1 million cells per mouse). On post-injection Day 5, SE (0.02 mL, 109 cells) was injected into the finger-pad of the right hindleg of control and test mice. The left hindleg was injected with 0.02 mL physiological saline solution. Local inflammatory reaction was assessed 18 h after the injections by comparison of the weights of both injected paws. CBA mice fed diet with protein concentrate derived from transgenic soybean, showed a reaction index (RI) of 23 ± 13. In CBA mice fed the conventional protein concentrate the RI was 41 ± 15; the control CBA mice fed a soy-free diet the RI was 30 ± 11. A similar trend was observed with C57Bl/6 mice, corresponding RI parameters were 48 ± 18 (40-3-2), 59 ± 24 (conventional soybean), and 51 ± 21 (control). These data show that

soybean line 40-3-2 produces no effect on the cellular component of the immune system compared to control mice.

Effect of soybean 40-3-2 on susceptibility to *Salmonella typhimurium* was investigated in mice. Mice fed diets supplemented with protein concentrate derived from conventional or transgenic soybean for four weeks were subsequently injected intraperitoneally with various doses of *Salmonella typhimurium* strain 415. The injected doses ranged from 10 to 105 microbial cells per mouse and varied on a 10-fold basis. The post-injection observation period was 14 days. The lifetime of the mice in the test group was somewhat longer than that of the control mice: the test mice infected with 105 or 104 microbial cells lived 4.2 and 6.2 days as compared with 1.2 and 2.2 days of the control mice, correspondingly. The smaller doses did not reveal any difference in the lifetime of mice in both groups. These data showed that *Salmonella typhimurium* produced typical infection both in control mice fed diet with conventional soybean protein concentrate and in the test mice fed diet with transgenic protein concentrate. According to the difference in the time to death, the test group took longer to die than the controls, although the differences in LD_{50} values remained within the experimental error. Thus, introduction of protein concentrate derived from transgenic soybean line 40-3-2 into mouse diet produced no effect on the humoral and cellular components of the immune system, did not sensitise the mouse organism, and did not disturb the natural resistance against typical infection such as murine typhus. Taken together, these data support the conclusion that transgenic soybean line 40-3-2 has no immunomodulating properties.

The potential impact of soybean 40-3-2 on systemic anaphylaxis was investigated in rats. The model of systemic anaphylaxis was according to standard protocols as described in the Russian Methodical Guidelines (MUK 2.3.2.970-00 (2000)). The study was performed on male Wistar rats (n = 49) weighing 180 ± 10 g. After a 7-day adaptation period to standard vivarium diet, the rats were fed a diet supplemented with protein concentrate (3.3 g/day/ rat) derived from conventional soybean (control group) or from soybean line 40-3-2 for 28 days. On experimental days 1, 3 and 5, the rats were sensitised intraperitoneally with 100 µg ovalbumin from hens' eggs (OVA). On Day 21, another portion of 10 µg OVA was administered under the same conditions to induce the secondary immune response. After termination of feeding animals with the diets on experimental Day 29, blood (0.2 mL) was drawn from the tail vein in order to assess the response of antibodies. Then a booster dose of OVA (30 mg/kg in 0.5 mL isotonic apyrogenic 0.15 M NaCl saline) was injected intravenously. During the following 24 h, the development of symptoms of active anaphylactic shock was observed. Severity of anaphylactic shock was scored as follows: +(1), shiver, chill, dyspnea; ++(2), asthenia, ataxia, peripheral cyanosis; +++(3), convulsions, paralysis; ++++(4) fatal outcome. The anaphylactic index (AI) was calculated according to the Russian Methodical Guidelines (MUK 2.3.2.970-00, 2000) as the mean of anaphylactic severity scores in a group at 24 h after injection of the booster dose. Intensity of humoral immune response was assessed according to concentration of circulating specific

immunoglobulin antibodies (the sum of IgG1 and IgG4 fractions) by the method of indirect solid-phase enzyme-linked immunosorbent assay (standard ELISA) on polystyrene. Results showed that the differences between the rats fed diets with protein concentrate derived from conventional soybean (control group) or line 40-3-2 were insignificant ($p>0.05$). There was only an insignificant trend to moderation of anaphylactic reaction in the 40-3-2 group. The antibody concentration did not significantly differ between the groups ($p>0.05$). The intensity of humoral immune response in the rats fed diet with protein concentrate derived from line 40-3-2 demonstrated a declining trend in comparison with the control group. The degree of sensitisation by ovalbumin in these rats did not increase compared with the rats fed diet with protein concentrate derived from conventional soybean. It was concluded from the study that the protein concentrate prepared from transgenic soybean line 40-3-2 did not elevate allergic reactivity and sensitisation towards the model allergen in test rats in comparison with the control rats fed conventional soybean.

Assessment of possible sensibilisation of 40-3-2 on the immune response to endogenous metabolic products was carried out by testing sensitivity to histamine in mice . For 21 days, the control and test mice were fed diets with protein concentrate derived from conventional and transgenic soybean. Then the mice of both groups were injected intraperitoneally with 2.5 mg histamine hydrochloride dissolved in 0.5 mL physiological saline solution. Twenty-four hours post-injection, all mice were alive.

Study on chronic (150 days) toxicity

A feeding study over 150 days with soy protein concentrate was conducted on male Wistar rats and biochemical, hematological, and morphological parameters were monitored in accordance with the requirements of the Ministry of Health of the Russian Federation authorised for risk and safety assessment of food derived from GM sources. Male Wistar rats ($n = 60$) with an initial body weight of 80–100 g were randomised into two groups. The test rats were provided daily with protein concentrate derived from the transgenic soybean line 40-3-2 (1.25 g per animal). The control rats were provided with the same amount of protein concentrate prepared from the conventional counterpart. The amount of the daily diet was 40.5 g per animal. Samples were collected on days 30 and 150 of the experiment. The absolute and relative weights and visual inspection of internal organs did not reveal any differences between the two groups. The histological assessments of internal organs (liver, kidneys, lung, spleen, small intestine, and testicle) revealed no differences between the control and test groups. The content of total protein, glucose, activity of alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase in blood serum, pH and the relative density of urine, urinary concentration of creatinine and its urinary excretion did not significantly differ between control and test rats at day 30 and 150. Hematological assays showed that feeding rats with protein concentrate derived from transgenic soybean line 40-3-2 did not induce significant changes in concentration of hemoglobin, hematocrit, total erythrocyte count, MCH, MCHC, MCV, total leukocyte count, absolute and relative count

of eosinophils, neutrophils, and lymphocytes relative to the control values obtained at 30 and 150 days.

Studies on reproduction and development

The potential effect of transgenic soybean 40-3-2 on pre- and postnatal development was investigated in Wistar rats. The experiments were performed on male and female Wistar rats as described in standard protocols (MUK 2.3.2.970-00 (2000)). The diet of the control rats comprised the protein concentrate derived from conventional soybean, while that of test rats included similar concentrate prepared from the transgenic soybean line 40-3-2 (1.25 g/ rat/day). The weight of the rats at mating was 280–350 g. The males and females were given soybean protein concentrate during the entire duration of the experiment, which included a 20-days preliminary diet, the mating period, the total time of gestation, and the entire period of lactation. The progeny was fed a diet with soybean protein concentrate ad libitum for 1 month after birth. To examine the potential embryotoxic effect of the soybean, 8 pregnant rats were sacrificed on Day 20 of gestation. The fetuses were extracted from the uteri and visually inspected. The corpus lutea and resorbed or dead embryos were counted. The overall pre- and post-implantation mortalities of the embryos were calculated. The craniocaudal size and weight of the fetuses were measured, and their sagittal histological sections were examined.

To study postnatal development, the progeny of 10 female rats in each group were examined for: the number of pups delivered by one female, the body weight of these pups (measured every week), the terms of total unfolding of the external ears, eye opening, fur development, eruption of incisors, and survival of progeny during 30 days. During the experiment, the general condition of the males, females, and progeny was satisfactory in both groups.

Comparison of the parameters of prenatal development of the progeny revealed no significant differences between the control and test groups of rats in terms of total embryonic mortality, the pre- and post-implantation embryonic mortality, and the size and weight of the fetuses. All these parameters varied within the physiological boundaries characteristic of Wistar rats. Examination of a series of sagittal sections revealed no abnormalities in the fetal development. The number of rat pups delivered by each female did not significantly differ between test and control groups in either the first or second generation. During the entire growth period, the body weight of the infant rats in either the first or second generation of the test group did not significantly differ from that of the control group. Survival of the newborn rats on Day 30 did not differ between test and control groups of rats in generations 1 and 2.