Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

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# Final health and environmental risk assessment of genetically modified soybean A2704-12

**Scientific opinion on herbicide tolerant, genetically modified soybean A2704-12 from Bayer CropScience for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (Application EFSA/GMO/NL/2005/18)**

**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) 2015: 07
Food/feed and environmental risk assessment of herbicide tolerant genetically modified soybean A2704-12 for food and feed uses, import and processing under Regulation (EC) No 1829/2003 (EFSA/GMO/NL/2005/18)

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

Dagrunn Engeset (VKM staff) and Inger Therese Lillegaard (VKM staff) are acknowledged for their valuable contribution to this scientific opinion [Chapter 4].

## Competence of VKM experts

Persons 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 A2704-12 expresses the phosphinothricin-N-acetyltransferase (pat) gene, from the soil bacterium *Streptomyces viridochromogenes*. The encoded PAT protein confers tolerance to the active herbicidal substance glufosinate-ammonium. Bioinformatics analyses of the inserted DNA and flanking sequences in soybean A2704-12 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 pat gene have been shown over several generations of soybean A2704-12. With the exception of the intended changes caused by the transgenetically introduced trait, data from field trials performed in the USA and Canada show that soybean A2704-12 is compositionally, morphologically and agronomically equivalent to its conventional counterpart and to other commercial soybean varieties. A repeated dose toxicity study in with rats and a nutritional assessment trial with broilers indicate that soybean A2704-12 is nutritionally equivalent to and as safe as conventional soybean varieties. The PAT protein produced in soybean A2704-12 does not show sequence resemblance to known toxins or IgE-dependent 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 discernible safety concerns associated with soybean A2704-12 regarding human or animal health or to the environment in Norway.

# 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 A2704-12 (Unique Identifier ACS-GMØØ5-3) from Bayer CropScience is approved under Regulation (EC) No 1829/2003 for food and feed uses, import and processing since 8 September 2008 (Application EFSA/GMO/NL/2005/18, Commission Decision 2008/730/EC).

Soybean A2704-12 has previously been assessed as food and feed by the VKM GMO Panel commissioned by the Norwegian Food Safety Authority related to the EFSAs public hearing of the application EFSA/GMO/2005/18 in 2006 (VKM 2006).

The food, feed and environmental risk assessment of the soybean A2704-12 is based on information provided by the applicant in the application EFSA/GMO/NL/2005/18, 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 A2704-12 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. VKM 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 A2704-12 includes 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 or 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 A2704-12 is derived from the conventional soybean variety A2704, which was transformed using particle bombardment. Soybean A2704-12 expresses the phosphinothricin-N-acetyltransferase (*pat*) gene, from the soil bacterium *Streptomyces viridochromogenes*. The encoded PAT protein confers tolerance to the active herbicidal substance glufosinate-ammonium.

### 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 two full length functional copies of the *pat* gene are present in the soybean A2704-12 genome. Similarity searches in 2006, with databases of known toxins and allergens did not indicate any potential for production of harmful proteins or polypeptides caused by the genetic modification. Southern blot and segregation analyses show that the introduced gene elements are stably inherited and expressed over several generations, and consistent with the observed phenotypic characteristics of soybean A2704-12. The VKM GMO Panel concludes that the molecular characterisation of soybean A2704-12 does not indicate a safety concern.

### Comparative assessments

The VKM GMO Panel has considered the available literature on compositional data and found that except for intermittent variations, no biologically relevant differences exist between soybean A2704-12 and its corresponding control A2704 in the analyses of seeds and various processed food and feed commodities. Differences observed could generally be explained by natural variability, environmental influences and/or storage conditions. The data presented do not show unintended effects as a result of the genetic modification.

Based on current knowledge, the VKM GMO Panel concludes that with the exception of the introduced trait, soybean A2704-12 is compositionally, agronomically and morphologically equivalent to its conventional counterpart and other conventional soybean varieties.

### Food and feed risk assessment

A 14-day repeated dose toxicity study with rats fed PAT protein, as well as a nutritional assessment trial with broilers fed diets containing soybean A2704-12 did not indicate any adverse effects. The PAT protein in A2704-12 does not show sequence resemblance to

known toxins or IgE-dependent allergens, nor has it been reported to cause IgE-mediated allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that soybean A2704-12 is nutritionally equivalent to and as safe as its conventional counterpart and other conventional soybean varieties.

### Environmental assessment

Considering the intended uses of soybean A2704-12, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure to microorganisms in the gastrointestinal tract and soil/water, mainly via intestinal content and faeces from animals fed feeds containing soybean A2704-12.

Soybean A2704-12 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 A2704-12. 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 is 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 and considering the intended usage, the VKM GMO Panel concludes that soybean A2704-12 is as safe as its conventional counterpart and other commercial soybean varieties. With the exception of the introduced trait, soybean A2704-12 is nutritionally, morphologically and agronomically equivalent to conventional soybean varieties.

Likewise, the VKM GMO Panel concludes that soybean A2704-12 does not represent a discernible environmental risk in Norway.

**Key words**: GMO, soybean (*Glycine max*), A2704-12, EFSA/GMO/NL/2005/18, herbicide tolerance, *pat,* 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 A2704-12 (unik kode ACS-GMØØ5-3) fra Bayer CropScience ble godkjent til import, videreforedling og til bruk som mat og fôr under EU-forordning 1829/2003 8. september 2008 (Kommisjonsbeslutning 2008/730/EU).

Soyalinjen A2704-12 ble første gang vurdert av VKMs faggruppe for GMO i 2006 (VKM 2006). Helserisikovurderingen ble utført på oppdrag av Mattilsynet i forbindelse med EFSAs offentlige høring av søknad EFSA/GMO/NL/2005/18.

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 A2704-12 har fått innsatt et *pat*-gen fra jordbakterien *Streptomyces viridochromogenes*. Genet koder for enzymet fosfinotricin acetyltransferase (PAT), som acetylerer og inaktiverer glufosinat-ammonium, virkestoffet i fosfinotricin-herbicider av typen Finale® og Liberty ®. Fosfinotricin er et ikke-selektivt kontaktherbicid som hemmer glutaminsyntetase. Enzymet deltar i assimilasjonen av nitrogen og katalyserer omdanning av glutamat og ammonium til aminosyren glutamin. Hemming av glutaminsyntetase fører til akkumulasjon av ammoniakk, og til celledød i planten. De genmodifiserte soyaplantene vil derfor tolerere høyere doser av plantevernmidler med virkestoffet glufosinat-ammonium 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 A2704-12. Resultatene viser at to komplette og funksjonelle *pat* gen er integrert i genomet til soyalinjen. Homologisøk fra 2006, med databaser over kjente toksiner og allergener, indikerer at genmodifiseringen ikke har ført til en mulig produksjon av skadelige proteiner eller polypeptider i soya A2704-12. Southern blot og segresjonsanalyser viser at de introduserte genene er stabilt nedarvet og uttrykt over flere generasjoner, og i samsvar med de fenotypiske egenskapene til soya A2704-12. VKMs faggruppe for GMO konkluderer med at den molekylære karakteriseringen ikke indikerer noen helserisiko ved soya A2704-12.

### Komparative analyser

VKMs faggruppe for GMO har vurdert tilgjengelig litteratur vedrørende soya A2704-12 og funnet at det, med unntak av små tilfeldige variasjoner målt i bønner og noen prosesserte komponenter til bruk i mat og fôr, ikke foreligger biologisk relevante forskjeller mellom den genmodifiserte soyaen og dens kontroll. Forskjellene kan mest sannsynlig forklares av naturlig variasjon, miljøpåvirkning og/eller lagringsbetingelser. De rapporterte dataene viser ingen utilsiktede effekter som følge av genmodifiseringen

Ut i fra dagens kunnskap konkluderer VKMs faggruppe for GMO at soya A2704-12, med unntak av den introduserte egenskapen, er ernæringsmessig, agronomisk, og morfologisk vesentlig lik dens konvensjonelle motpart, samt andre konvensjonelle soyasorter.

### Helserisiko

En 14 dagers toksisitetsstudie med rotter gitt PAT-protein i fôret, og en ernæringsstudie utført med broilere gitt fôr inneholdende soya A2704-12, har ikke indikert helseskadelige effekter. PAT-proteinet viser ingen sekvenslikhet med kjente toksiner eller IgE-bundne allergener, og er heller ikke rapportert å ha forårsaket IgE-medierte allergiske reaksjoner.

Ut i fra dagens kunnskap konkluderer VKMs faggruppe for GMO at soya A2704-12 er ernæringsmessig lik, og like trygg som, dens konvensjonelle motpart og andre konvensjonelle sorter.

### Miljørisiko

Med bakgrunn i tiltenkt bruksområde for søknaden er miljørisikovurderingen av soyalinjen A2704-12 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 A2704-12 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 A2704-12, ved forskreven bruk, er like trygg som dens konvensjonelle motpart og andre konvensjonelle soyasorter. Soya A2704-12 er ernæringsmessig, morfologisk, og agronomisk ekvivalent med konvensjonell soya.

Likeledes finner faggruppen, ut i fra dagens kunnskap, at den omsøkte bruken av soya A2704-12 ikke vil medføre noen miljørisiko i Norge.

# Abbreviations and explanations

| | |
|---|---|
| **ADF** | Acid Detergent Fibre. The insoluble residue remaining after boiling a feed/food sample in acid detergent solution. It contains many insoluble (structural) fibre components – lignin, cellulose, silica – but also insoluble forms of nitrogen. It does not, however, contain hemicellulose. See also NDF. |
| **Aspirated grain fractions** | Plant parts obtained during normal aspiration of cereal and oil seed crops in the handling of the product consisting primarily of plant parts, including glumes and contain not more than 15 percent ash (dirt), The American Feed Control Officials definition |
| **ARMG** | Antibiotic resistance marker gene |
| **BC** | Backcross. Backcross breeding 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 |
| **MS** | Member States |
| **NFSA / 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 |
| **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 |

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|---|---|---|
| **OSR** | Over season root | |
| **OSWP** | Over season whole plant | |
| ***pat*** | *Phosphinothricin-Acetyl-Transferase* gene | |
| **PAT** | Phosphinothricin-Acetyl-Transferase *protein* | |
| **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** | Transgene copy number is defined as the number of exogenous DNA | |

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|---|---|
| **number** | 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

On 13 July 2005, the European Food Safety Authority (EFSA) received from the Competent Authority of the Netherlands an application (Reference EFSA/GMO/NL/2005/18) for authorisation of the genetically modified herbicide tolerant soybean A2704-12 (Unique Identifier ACS-GMØØ5-3), submitted by Bayer CropScience within the framework of Regulation (EC) No 1829/2003.

The scope of the application covers:

- Food
  - ✓ GM plants for food use
  - ✓ Food containing or consisting of GM plants
  - ✓ Food produced from GM plants or containing ingredients produced from GM
  - ✓ Plants
- Feed
  - ✓ GM plants for feed use
  - ✓ Feed containing or consisting of GM plants
  - ✓ Feed produced from GM plants
- GM plants for environmental release
  - ✓ Import and processing (Part C of Directive 2001/18/EC)

After receiving the application EFSA/GMO/NL/2005/18 and in accordance with Articles 5(2)(b) and 17(2)(b) of Regulation (EC) No 1829/2003, EFSA informed the EU- and EFTA Member States (MS) and the European Commission and made the summary of the dossier publicity available on the EFSA website. EFSA initiated a formal review of the application to check compliance with the requirements laid down in Articles 5(3) and 17(3) of regulation (EC) No 1829/2003. On 10 February 2006, EFSA declared the application 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), following the requirements of Articles 6(4) and 18(4) of Regulation (EC) No 1929/2003, 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 application under assessment. The VKM GMO Panel assessed the application in connection with the EFSA official hearing, and submitted a preliminary opinion in May 2006 (VKM 2006). EFSA published its scientific opinion 3 July 2007 (EFSA 2007), and soybean A2704-12 was approved for food and feed uses, import and processing 8 September 2008 (Commission Decision 2008/730/EC).

# Terms of reference

The Norwegian Environment Agency (formerly the 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 upon 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 upon the deliberate release of GMOs pursuant to the Gene Technology Act and the Food Safety Act. In addition, 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 VKM, 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 VKM already has conducted its final risk assessments on. However, the Norwegian Environment Agency requests VKM 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 NFSA to give final opinions on all 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.

NFSA has therefore, by letter dated 13 February 2013 (ref. 2012/150202), requested 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 GMOs 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 and 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 NFSA. In addition, the changes related to herbicide residues of GMPs 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 A2704-12 (Unique Identifier ACS-GMØØ5-3) was developed to provide tolerance to the herbicidal active substance glufosinate-ammonium by the introduction of a gene coding for the phosphinothricin N-acetyltransferase enzyme (PAT) from the soil bacterium *Streptomyces viridochromogenes*.

Glufosinate-ammonium inhibits glutamine synthetase, leading to glutamine deficiency, ammonia accumulation and eventually to plant death. The PAT protein catalyses the conversion of glufosinate-ammonium to N-acetyl glufosinate. N-acetyl glufosinate is an inactive form that does not bind to glutamine synthetase allowing plants to grow in the presence of glufosinate-ammonium.

The genetic modification in soybean A2704-12 is intended to improve agronomic performance only and is not intended to influence the nutritional properties, the processing characteristics or the overall use of soybean as a crop.

Soybean A2704-12 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.

VKM 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 A2704-12 is based on information provided by the applicant in the application EFSA/GMO/NL/2005/18, 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

Particle bombardment was used to transform embryo shoot apices derived from the soybean cultivar A2704 to generate the glufosinate-ammonium tolerant event A2704-12. DNA fragments of the plasmid pB2/35SAcK were used in the transformation. A summary of molecular studies of soybean A2704-12 is shown in Table AI-1, Appendix.

### 2.1.2 Nature and source of the vector used for the transformation

The plasmid pB2/35SAcK (~ 4kb) is a derivative of the vector pUC19. It contains a Right Border fragment (RB) from the *Agrobacterium tumefaciens* Ti plasmid pTiAch5 and a synthetic *pat* gene inserted between a 35S-promotor (P35S) and 35S-terminator (T35S) from Cauliflower Mosaic Virus (CaMV) (Berghman & De Beuckeleer, 2002a).

The plasmid vector also contains the *β-lactamase* (*bla*) gene which confers resistance to the antibiotic ampicillin, and the bacterial origin of replication (*ori*) from vector pUC19. Prior to transformation, plasmid pB2/35SAcK was digested with the restriction enzyme *Pvu*I to disrupt the coding sequence of the *bla* gene and thereby remove the possibility of its expression. A plasmid map of pB2/35SAcK is shown in Figure 2.1.2-1, and Table 2.1.2-1 indicates the relative position and function of the genetic elements in the plasmid.

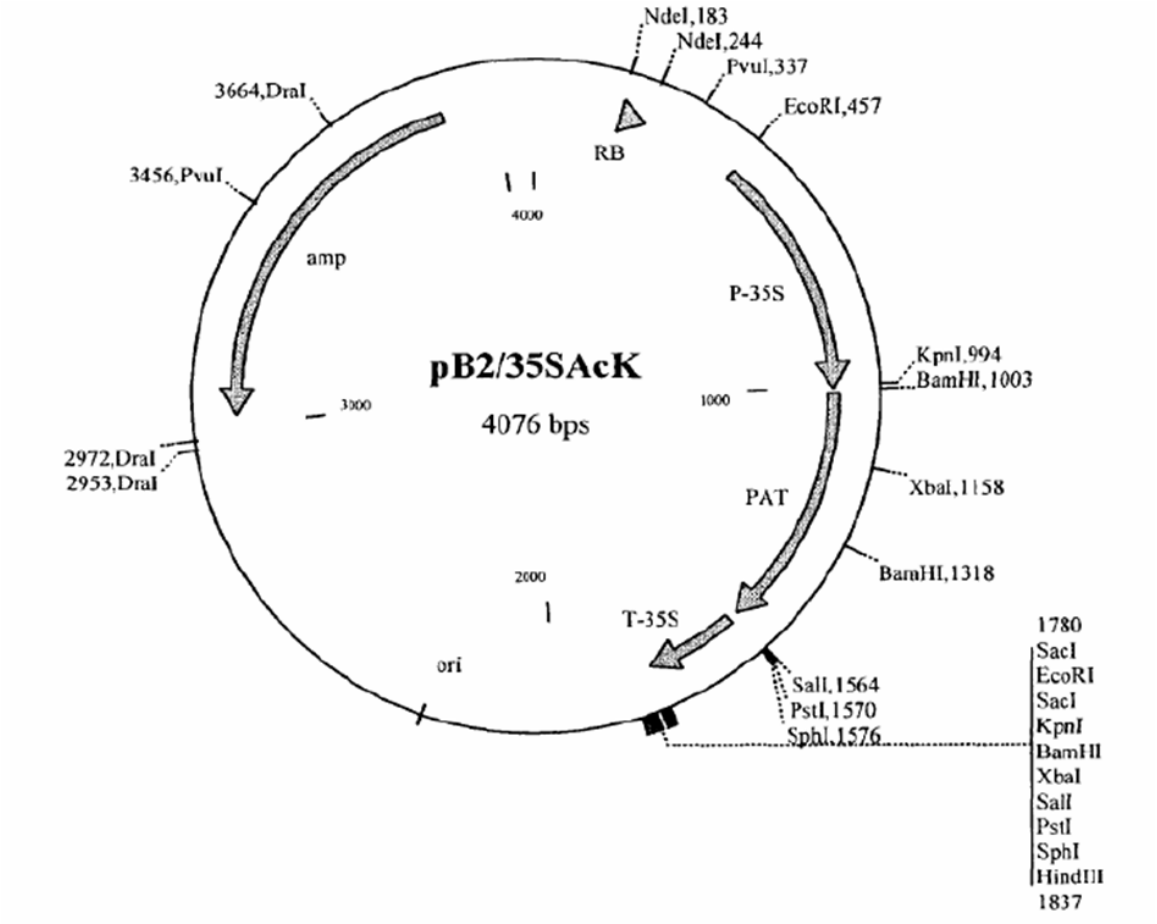


**Figure 2.1.2-1.** Plasmid map of pB2/35SAcK (Figure 5 in Technical dossier)

**Table 2.1.2-1.** Genetic elements of the plasmid pB2/35SAcK (Table 4 in Technical dossier)

| Position in vector | Genetic element |
|---|---|
| 0001-0188 | Sequence of the vector pUC19 (Yanisch-Perron *et al.*, 1985) |
| 0189-0243 | **RB: Right Border** fragment of octopine plasmid TiAch5 (Gielen *et al.*, 1984) |
| 0244-0460 | Sequence of the vector pUC19 (Yanisch-Perron *et al.*, 1985) |
| 0461-1003 | **P35S: promoter** from Cauliflower mosaic virus from the vector PDH51 (Pietrzak *et al.*, 1986) |
| 1004-1011 | Synthetic polylinker derived sequences |
| 1012-1563 | *pat:* Synthetic *pat* gene (amino acid sequence from *Streptomyces viridochromogenes*) (Strauch *et al.*, 1993) |
| 1564-1581 | Synthetic polylinker derived sequences |
| 1582-1784 | **T35S: terminator** from Cauliflower Mosaic Virus from the vector pDH51 (Pietrzak *et al.*, 1986) |
| 1785-4076 | Sequence of the vector pUC19, including the polylinker (pos. 185-1843), the **ori** (origin of replication) at position 2257 and the ***β-lactamase* (*bla*) gene** (pos. 3876-3016) (Yanisch-Perron *et al.*, 1985) |

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

The right border repeat, RB, is a fragment of the octopine plasmid TiAch5 and facilitates the incorporation of the T-DNA to the receiving genome. The modified *pat* gene is derived from the bacterium *Streptomyces viridochromogenes*, a gram positive sporulating soil bacterium. The modified *pat* gene encodes the enzyme phosphinothricin acetyl transferase (PAT) which confers tolerance to glufosinate-ammonium based herbicides by acetylating glufosinate into a non-phytotoxic metabolite. The 35S promoter and 35S terminator from CaMV are derived from the vector PDH51, and direct constitutive expression of the *pat* gene and termination of transcripts, respectively. *β-lactamase* (*bla*) is an antibiotic resistance gene used as a bacterial marker. Due to digestion by the restriction enzyme *Pvu*I in the coding sequence of the *bla* gene, it is not functional in soybean A2704-12.

## 2.2 Information relating to the GM plant

### 2.2.1 Description of the trait(s) and characteristics introduced or modified

Soybean A2704-12 contains two functional copies of the *pat* gene cassette at a single locus. The *pat* genes encode the enzyme phosphinothricin acetyl-transferase (PAT), which metabolises glufosinate to an inactive, acetylated derivative, thereby conferring tolerance to glufosinate-ammonium herbicides.

The native bacterial *pat* gene has a high G:C content, which is not typical of plant genes. To improve expression of *pat* in soybean A2704-12, a synthetic version with a lower G:C content was therefore constructed for the development of A2704-12. This modified *pat* gene has approximately 70% DNA sequence identity with the native *pat*. According to the applicant this modification did not alter the encoded amino acid sequence of the PAT protein.

### 2.2.2 Information on the sequences actually inserted or deleted

Molecular analyses were conducted to determine the nature, number, integrity and stability of the DNA insert in soybean A2704-12. Genomic DNA was analysed by Southern blot and DNA sequencing 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/locus).

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

The DNA insert in A2704-12 has a length of 6780 bp and its sequence is described in its entirety in Berghman & De Beuckeleer (2002). Table 2.2.2-1 describes all sequences actually inserted in soybean event A2704-12.

The applicant has performed extensive Southern blot analyses. These analyses were conducted on isolated genomic DNA from leaf tissues of soybean A2704-12 and controls, digested with the seven restriction enzymes *EcoR*I, *Sph*I, *Nco*I, *Hind*III, *BamH*I, *EcoRV*, and *Dra*I. Genomic DNA from the nontransgenic parent cultivar A2704, and A2704 + plasmid pB2/35SAcK, were used as negative and positive controls, respectively. Four types of PCR-generated probes were used. According to the applicant the hybridisation patterns from these analyses show that two copies of the *pat* gene sequence are inserted into the plant genome at a single site in a head-to-tail configuration (Figure 2.2.2.1-1a & 2.2.2.1-1b). Between the two *pat* copies, one copy of the 3' *bla* sequence and one copy of the 5' *bla* sequence are integrated in a reverted orientation to each other. This is supported by the observed hybridisation patterns and DNA sequence analyses. Additionally, a short fragment (27 bp) of the 3' *bla* sequence is also inserted after the second *pat* gene (Table 2.2.2-1). According to the applicant the inserted *bla* gene sequences do not constitute an intact *bla* gene, because of their inverted orientation (Figure 2.2.2.1-1a & 2.2.2.1-1b). A more detailed description of the results can be found in De Beuckeleer & Botterman (1999) and on pages 35-49 in the Technical dossier. Figure 2.2.2.1-1a shows a schematic representation of restriction sites, insert and genomic flanking sequences in soybean A2704-12.

**Table 2.2.2-1.** Description of the sequences actually inserted in soybean event A2704-12 (Table 10 in Technical dossier)

| Nucleotide Number | Corresponding nucleotide number in pB2/35SAcK | Genetic element |
|---|---|---|
| 1 --> 121 | 340 --> 460 | Sequence of vector pUC19 |
| 122 --> 664 | 461 --> 1003 | **35S promoter** from Cauliflower Mosaic Virus from vector pDH51 |
| 665 --> 672 | 1004 --> 1011 | Polylinker sequences |
| 673 --> 1224 | 1012 --> 1563 | **Synthetic *pat* gene** |
| 1225 --> 1242 | 1564 --> 1581 | Polylinker sequences |
| 1243 --> 1445 | 1582 --> 1784 | **35S terminator** from Cauliflower Mosaic Virus from vector pDH51 |
| 1446 --> 2676 | 1785 --> 3015 | Sequence from vector pUC19, including the polylinker and the **origin of replication** (not functional in plants) |
| 2677 --> 3122 | 3016 --> 3461 | **Part of** the complementary sequence of the ***beta-lactamase* gene** (bp 861 <-- bp 416 of the coding sequence) |
| 3123 --> 3458 | 336 --> 1 | Sequence of vector pUC19 |
| 3459 --> 3658 | 4076 --> 3877 | Sequence of vector pUC19 |
| 3659 --> 4073 | 3876 --> 3462 | **Part of** the complementary sequence of the ***beta-lactamase* gene** (bp 1 --> bp 415 of the coding sequence) |
| 4074 --> 4197 | 337 --> 460 | Sequence of vector pUC19 |
| 4198 --> 4740 | 461 --> 1003 | **35S promoter** from Cauliflower Mosaic Virus from vector pDH51 |
| 4741 --> 4748 | 1004 --> 1011 | Polylinker sequences |
| 4749 --> 5300 | 1012 --> 1563 | **Synthetic *pat* gene** |
| 5301 --> 5318 | 1564 --> 1581 | Polylinker sequences |
| 5319 --> 5521 | 1582 --> 1784 | **35S terminator** from Cauliflower Mosaic Virus from vector pDH51 |
| 5522 --> 6752 | 1785 --> 3015 | Sequence from vector pUC19, including the polylinker and the **origin of replication** (not functional in plants) |
| 6753 --> 6780 | 3016 --> 3043 | **Part of** the complementary sequence of the ***beta-lactamase* gene** (bp 861 <-- bp 834 of the coding sequence) |

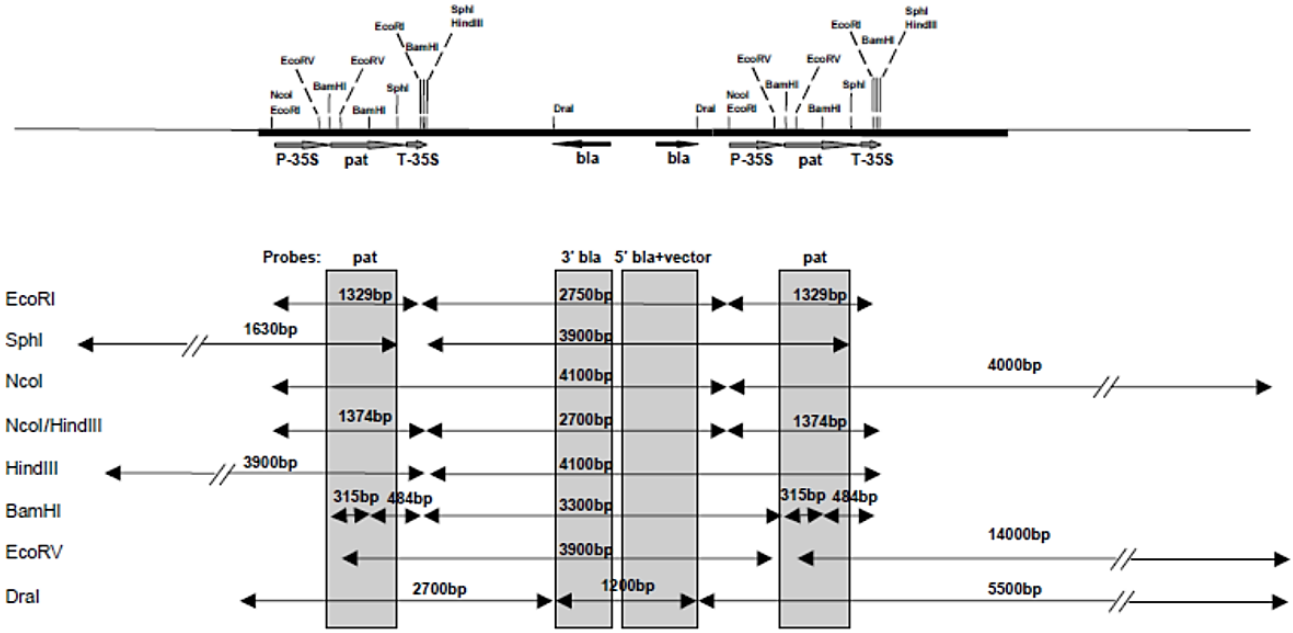


**Figure 2.2.2.1-1a.** Schematic representation of the insert sequence in soybean A2704-12, with restriction sites used in the Southern blot analyses (Figure 7. In Technical Dossier).

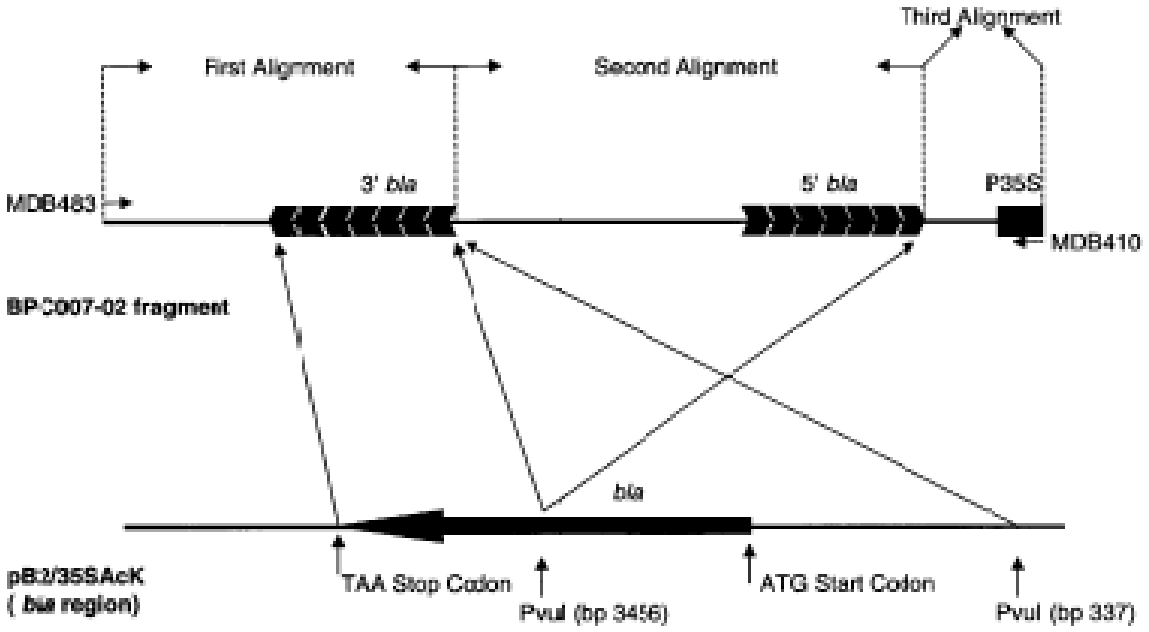


**Figure 2.2.2.1-1b.** Schematic representation of the insert sequence in soybean A2704-12, showing the inverted orientation of the small PvuI fragment (from Berghman & De Beuckeleer 2002b).

#### *2.2.2.2 The organisation of the inserted genetic material including its sequence data and that of the flanking 5' and 3' regions*

Based on the result of the completeness check, the applicant was requested by EFSA to provide additional information on the molecular characterisation of soybean A2704-12. Updated information was submitted by the applicant on 2 August, 2006. This update included extended sequence data of the 5' flanking region. The first submission by Bayer CropScience provided the flanking sequence information at the 5' and 3' site of the inserted DNA cassette with 198 bp and 299 bp respectively. In the study by Moens & Habex (2006a), the applicant established by means of Southern blot and PCR analyses that a substantial amount of chloroplast DNA sequence is inserted in the soybean genome, and estimated that between 2510 and 2718 bp of the 5 flanking sequences derive from integrated chloroplast DNA. In their final study (Moens & Habex, 2006b), 4044 bp of the 5' flanking sequences were determined, indicating that a total length of the inserted chloroplast DNA equals 2566 bp.

#### *2.2.2.3 In the case of deletion(s), size and function of the deleted region(s)*

An updated analysis in 2006 revealed a deletion of 2082 bp from the soybean genomic DNA at the insertion site.

The applicant has performed a bioinformatic analysis including both flanking regions and the pre-insertion locus in order to predict the effect of the deletion (Bates, 2006). DNA similarity searches to the regions of the wild type A2704 soybean showed matches with *Arabidopsis* expressed sequence tags (EST). Two putative gene products had significant similarities:

According to the applicant one of these loci is located entirely within the 5' flanking region and is unlikely to be affected by the transgene insertion. It shows strong homology to an *Arabidopsis thaliana* clone of unknown function and a weak homology to histone-like DNA transcription factors. The second putative gene is situated at a pre-insertion locus and is partially deleted by the transgene insertion. According to the applicant the function of similar genes in *Arabidopsis* is unknown, and that homologies to hypothetical proteins in dicotyledonous plants and to nucleotide-binding family proteins suggest that this putative gene is a transcription factor, represented in *Arabidopsis thaliana* as a small multi-gene family.

According to the applicant, agronomic performance and nutritional impact studies suggest that the reported findings do not cause pleiotropic effects in the soybean plant, indicating that this putative protein is not essential, or that another member of the possible multi-gene family compensates for the deletion.

### 2.2.3 Information on the expression of the inserted sequence

PAT protein levels have been measured in samples of root, stem, leaf and seed of soybean A2704-12 from greenhouse trials and field trials conducted in the US and Canada. Table 2.2.3-1 summarises the level of PAT detected in samples harvested from 12 different field locations, over a period from 1996 through 1999. In all cases, the level of PAT was determined with an enzyme-linked immunosorbent assay (ELISA) using PAT specific antibodies. Some field trials included comparisons of soybean A2704-12 treated with glufosinate-ammonium herbicide (Liberty®) to A2704-12 not treated with the herbicide. Overall, from the analyses represented in Table 2.2.3-1, the level of PAT was found to range from 0.48 – 2.4 µg/g fresh weight (fw) (478 - 2382 ng/g) of seed. The PAT content of seed, averaged over locations, was not significantly influenced by herbicide application.

**Table 2.2.3-1.** Summary of PAT protein levels in seed of soybean A2704-12 from several field trials conducted in USA and Canada (Table 12 in Technical dossier)

| Trial Location & Year of Trial (Reference Code) | PAT ng /g sample (conventional treatment) | PAT as % of crude protein | PAT ng /g sample (Liberty sprayed) | PAT as % of crude protein |
|---|---|---|---|---|
| Webster City, IA 1996 (A) | 524 - 622[1)] | 0.00016%[2)] | | |
| Webster City, IA 1996 (B) | 503 – 647[3)] | 0.00016% | | |
| Bethany, IL 1996 (B) | 1516 – 1934[3)] | 0.00045% | | |
| York, NE 1996 (B) | 816 – 1078[3)] | 0.00025% | | |
| Seymour, IL 1999 (C) | 1105 – 1141[4)] | | 1153 – 1194[4)] | |
| York, NE 1999 (C) | 1051 – 1213[4)] | | 923 – 1139[4)] | |
| Waterloo, WI 1999 (C) | 648 – 732[4)] | | 723 – 749[4)] | |
| Branchton, Ontario 1999 (C) | 478 – 572[4)] | 0.000227%[5)] | 486 – 614[4)] | 0.000227%[5)] |
| Seymour, IL, 1999 (D) | 2138 | 0.00050[6)] | 1948 | 0.00056[6)] |
| Sarnia, Ontario 1997 (E) | NA | | 1214 – 1320[3)] | |
| Dover Centre, Ontario 1997 (E) | 1108 – 1647[3)] | | 1004 – 1614[3)] | |
| Harrow, Ontario 1997 (E) | 1494 – 1713[3)] | | 1512 – 2382[3)] | |

(A) Shillito, 1997 (B) Barraj, 1998 (C) Shillito, 2001b (D) Shillito, 2003a (E) Cromar, 1999

[1)]Range from two extractions; [2)] Calculated by using the highest level of PAT or the only level of PAT and the lowest level of crude protein reported in the literature for soybean seed (grain) (37%); [3)] Range from 3 replicates, two extractions each; [4)] Range from 3 replicates; [5)] Calculated by using the average PAT content across the four sites and the average level of crude protein in the samples; [6)] Calculated using measured crude protein levels in samples; NA – samples were not available for analysis

The level of PAT protein in leaves was measured at four different early growth stages of soybean A2704-12 grown in a single greenhouse trial in USA in 2002 (Scott & Currier, 2003).

A2704-12 plants were either sprayed once or twice with Liberty® at an application rate of 0.35 pounds active ingredient per acre, or not sprayed with Liberty®. Plant samples were taken for analysis at the V3, V5-6, V6-7 and V8 vegetative (V) growth stages. Bloom generally occurs at the V7 – V10 growth stages. The average amount of PAT protein measured in the four growth stages ranged from 8.5 µg/g to 28.2 µg/g (fw). PAT protein comprised an average of 0.010 – 0.035% of the total crude protein in the leaves of soybean event A2704-12. Table 2.2.3-2 indicates the average quantities of PAT at the different stages.

**Table 2.2.3-2**. Summary of PAT protein levels in soybean leaves collected from A2704-12 at different growth stages, treated and not treated with glufosinate-ammonium herbicide (Liberty®) (Table 11 in Technical dossier)

| Genotype | Liberty Treated | PAT protein content (µg/g ± SD) on a fresh weight basis at specified growth states (V3-V8) | | | |
|---|---|---|---|---|---|
| | | V3 | V5-6 | V6-7 | V8 |
| A2704 | No | ND | ND | ND | ND |
| A2704-12 | No | 13.5 ± 7.2 | NA | NA | NA |
| A2704-12 | No | 8.4 ± 5.12 | 20.2 ± 2.9 | 14.2 ± 11.7 | 28.2 ± 5.0 |
| A2704-12 | Once | NA | 14.3 ± 7.3 | 19.3 ± 2.8 | 17.9 ± 13.2 |
| A2704-12 | Twice | NA | NA | NA | 25.0 ± 1.5 |

SD = Standard Deviation, ND = Not Detectable, NA = Not Applicable, V = Vegetative Growth Stage (from emergence to flowering) Vn : n is the number of nodes on the main stem with fully developed leaves. V3 stage occurs about 25 days after planting. V7 stage occurs about 40 days after planting.

PAT levels measured in roots, stems and leaves ranged from 0.30 – 3.69 µg/g, 4.86 – 10.0 µg/g and 11.7 – 17.6 µg/g (fw), respectively, in samples from soybean A2704-12 grown in a greenhouse study. The plants were not sprayed with Liberty® herbicide, and were sampled at the V2 - V4 growth stage (Currier, 2003). The average PAT contents are summarised in Table 2.2.3-3. The levels found represent 0.011%, 0.021% and 0.024% of the total crude protein in roots, stems and leaves, respectively.

**Table 2.2.3-3.** Summary of PAT protein levels in roots, stems and leaves collected from soybean A2704-12 at growth stages V2-V4 (Table 13 in Technical dossier)

| Matrix | Average PAT protein content (µg/g) ± SD | Crude Protein as % Fresh Weight | PAT Protein content as %Crude Protein |
|---|---|---|---|
| Root | 2.23 ± 1.29 | 1.95 | 0.011 |
| Stem | 7.63 ± 2.20 | 3.58 | 0.021 |
| Leaf | 14.5 ± 2.4 | 3.90 | 0.024 |

SD = Standard Deviation

The applicant has also performed a Northern Blot analysis in order to determine if any of the two *bla* sequences present in soybean A2704-12 are expressed. Plant RNA was extracted from seeds, leaf, root and stem tissues, separated according to size and transferred to a membrane. The membrane was probed with radioactive labeled anti-sense *bla* RNA and measured by autoradiography. *In vitro* synthesised sense *bla* RNA served as reference substance. The analysis showed that none of the *bla* sequences were expressed in the tested plant tissues (De Beuckeleer & Botterman, 1997).

### *2.2.3.1 Part of the plant where the insert is expressed*

Production of the PAT protein is expected to occur throughout the whole plant since the 35S promoter (P35S) from the CaMV is considered to drive constitutive expression. However, as seen from the protein levels reported in Table 2.2.3-1 and 2.2.3-2 shown above, there are some natural biological variations related to different plant tissues.

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

In the report by Berghman (2005) in the original submission, seven open reading frames (ORFs) were defined as newly created or chimeric ORFs: ORF-1 and ORF-2 were newly created at the 5-prime flanking chloroplast/insertion DNA junction of soybean A2704-12. ORF-3, ORF-4, ORF-5, ORF-6 and ORF-7 were created over the *Pvu*I digested junction fragments. No newly created ORFs were detected at the 3-prime flanking/insertion DNA junction of soybean A2704-12. An updated bioinformatic analysis was performed by the applicant in 2006 (De Pestel, 2006) for the new junction region of the chloroplast and the genomic DNA mentioned in 2.2.2.4. The analysis showed one additional newly created putative amino acid sequence (ORF8). An updated bioinformatics analysis of all putative ORFs was performed by the applicant in 2006 (Hérouet-Guicheney, 2006). According to the applicant, none of the 8 putative ORFs showed sequence identity with known toxins or allergens.

### 2.2.4 Genetic stability of the insert and phenotypic stability of the GM plant

#### *2.2.4.1 Genetic stability of the insert in soybean A2704-12*

To assess the genetic stability of the insert in soybean A2704-12, several studies have been performed by the applicant.

In a study by De Beuckeleer (1998), Southern blot analyses were used on DNA extracts from leaf samples of three successive generations (R3, R4 and R5) of soybean derived from the original transformant (R0) of A2704-12. The DNA samples were subjected to digestion with *Hind*III and *Nco*I, both enzymes having one restriction site in the transformation plasmid. The *pat* cassette sequence was used as probe in the analysis. The probe hybridised with the plasmid and upstream plant DNA sequences when the samples were digested with *Hind*III, and with plasmid and downstream plant DNA sequences when digested with *Nco*I. According to the applicant, the results of the analyses showed no difference in banding patterns between the samples, indicating genetic stability of the insert over three generations.

In a multigenerational study (Currier 2005), DNA samples were isolated from soybeans that contained the A2704-12 insert, and had either equal or different genetic backgrounds (from a series of crosses, backcrosses and selfings after an initial cross with A2704-12) and/or of different geographical origin.

Plants with different genetic backgrounds were grown in the field for 14 generations. Plants with similar genetic backgrounds were grown for 8 or 9 generations. Samples of the 14th generation came from plants that were all grown in the same final sampling location. However, during their development, they were bred in different geographical locations. Samples of the 8th and 9th generation of plants were taken from plants grown in the

greenhouse from seeds that had been produced at several different geographical locations. Genomic DNA was prepared from the leaves of five individual plants from each sampling location and soybean line. Next, the isolated DNA was digested with the restriction enzyme *EcoR*V, and probed with the 35S-PAT DNA sequence. *EcoR*V has four restriction sites within the A2704-12 insert, and two sites close to the right and left borders of the insert. Two of these sites are only 181 base pairs apart and are too small to produce a signal in a Southern blot. According to the applicant, digestion with *EcoR*V and probing with the 35S-PAT sequence should therefore give 3 bands of 3.4, 3.9 and 14 Kb in a Southern blot. According to the applicant, all of the analysed plants contained the expected banding patterns. The results indicate genetic stability of the A2704-12 DNA-insert, in soybeans of different genetic backgrounds grown for multiple generations, and in soybeans of the same genetic background grown at different locations.

#### *2.2.4.2 Phenotypic stability of the glufosinate-ammonium tolerant trait in A2704-12*

The applicant has assessed the phenotypic stability of soybeans derived from event A2704-12 by evaluating the inheritance patterns of glufosinate-ammonium tolerance through successive generations. The original (R0) hemizygous (*pat/-*) transformant plant was first self-pollinated to produce R1 progeny seeds, consisting of homozygous (*pat*/*pat*), heterozygous (*pat*/-) and homozygous non-transgenic (-/-) seeds (expected ratio of 1:2:1, respectively). The R1 progeny seeds were subsequently planted and the plants sprayed with glufosinate ammonium.

R2-seeds from the tolerant R1 plants (*pat*/*pat* and *pat/-*) were retained and planted in a plant to row fashion, i.e. rows were planted with seeds from one plant only. If the *pat* gene was inherited as a single dominant gene, plants in 1/3 of the rows should be fully tolerant (*pat*/*pat*) to glufosinate-ammonium, while 2/3 would have some plants that were tolerant (either *pat/pat* or *pat/-*) and some that were not (-/-). The results showed that 24 rows were fully tolerant and 45 rows were partially tolerant to glufosinate ammonium (expected ratio, 1:2), and that 67 individual plants were tolerant and 24 were not (expected ratio 3:1) (Table 2.2.4.2-1). These results were not significantly different from the expected ratios, and indicate that the *pat* gene expression is inherited in a Mendelian fashion consistent with a single dominant *pat* locus (VanWert, 1999).

**Table 2.2.4.2-1**. Segregation data for individuals and rows of progeny of self-pollinated event A2704-12 (Table 14 in Technical dossier).

| Parents and zygosity for the *pat* locus | Progeny | Fully Resistant Rows/ Plants | Partially Resistant Rows/ Sensitive Plants | Expected Ratio | $\chi^{2b}$ |
|---|---|---|---|---|---|
| Tolerant R1progenies of the self-pollinated R0 transformants (1/4 *pat*/*pat* ; 2/4 *pat*/-) | R2 Rows | 24 | 45 | 1:2 | 0.06 |
| Hemizygous R1 plants (*pat*/-) resulting in the partially resistant rows | R2 Individual Plants | 67 | 24 | 3:1 | 0.10 |

[b]No significant difference (p=0.05) for the Chi square goodness-of-fit test for hypothesis of either 3:1 or 1:2 segregation (Significance at p=0.0.5 for $\chi^2 \geq 3.84$, df = 1).

## 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 two full length functional copies of the *pat* gene are present in the soybean A2704-12 genome. Similarity searches in 2006, with databases of known toxins and allergens did not indicate any potential for production of harmful proteins or polypeptides caused by the genetic modification. Southern blot and segregation analyses show that the introduced gene elements are stably inherited and expressed over several generations, and consistent with the observed phenotypic characteristics of soybean A2704-12. The VKM GMO Panel concludes that the molecular characterisation of soybean A2704-12 does not indicate a safety concern.

# 3 Comparative assessments

## 3.1 Production of material for comparative assessments

For compositional studies, A2704-12 soybean was compared to the commercial non-transgenic parental variety A2704 (control) that is grown in the US because of its desirable agronomic performance. The field trials were carried out during the year 1999 in Illinois, Nebraska, Wisconsin (US) and Ontario (Canada) and during the year 2000 in Iowa, Indiana, Wisconsin, Minnesota (US) and Ontario (Canada), a total of nine plots. The plants were grown under conditions typical of commercial production practices. Three replicates were used for each of the three treatments at each trial site, in a randomised study design: non-GM A2704 soybean, GM A2704-12 soybean not glufosinate sprayed, GM A2704-12 sprayed with glufosinate ammonium. The fields were sprayed twice at the equivalent of 392 grams of active ingredient per hectare.

Nontreated hay and forage samples from soybean A2704-12 and its conventional, non-transgenic counterpart A2704 were grown at 3 different sites in the USA (Iowa, Nebraska and Illinois) in 1996. None of the soybeans at any of these sites were sprayed with glufosinate in the 1996 field study, while the soybean in 1999 and 2000 were sprayed with glufosinate.

**Statistical analysis**

All statistical analyses were performed using SAS version 8.2 (WINDOWS 98). A by-site analysis of differences was performed for each component. In the over-all site analysis, the variance was calculated (ANOVA) with treatment and site as interaction terms. T-tests were performed to compare non-transgenic A2704 with transgenic A2704-12 not sprayed (A versus B) and non-transgenic A2704 with transgenic A2704-12 sprayed with glufosinate (A versus C) in the single site analysis. EFSA requested that the applicant conduct a new statistical analysis, which was provided (Rattemeyer, 2006). None of the studies were performed according to EFSA's most recent guidelines (EFSA, 2011). However the studies were carried out prior to the publication of these guidelines.

## 3.2 Compositional analysis

### 3.2.1 Field trials performed in 1999 and 2000

Soybean seeds were collected for compositional analysis with respect to proximates, fibre compounds, micro-nutrients (minerals, vitamins), amino acids, fatty acids, anti-nutrients (i.e. phytic acid, trypsin inhibitors, lectins, stachyose and raffinose), and other secondary metabolites (isoflavones) (Technical dossier, Oberdörfer, 2003). The compounds analysed was in agreement with the recommendation by OECD (OECD, 2001). An additional analysis

(ANOVA) of compositional parameters has been provided by the applicant in response to a request by the EFSA GMO Panel (Rattemeyer, 2006). In addition to the analysis of soybean seeds, the applicant analysed hay, forage, hulls, untoasted meal, toasted meal, protein isolate, refined oil, and crude lecithin (Table AII-1).

The applicant has compared the compositional data in soybean A 2704-12 and A 2704 with standard composition data taken from the sources presented in Table AII-2.

*Proximate and fiber composition of harvested seeds*
In Table AII-3 the over-all site results for content of proximates in seeds are presented, together with standard composition data for soybean. All values are within the reference ranges found in the literature. The by-site analysis of all proximates except crude protein resulted in statistically significant differences ($p < 0.05$) between treatments for up to three of nine sites. For crude protein, comparing treatment A and C indicated statistically significant differences for a total of six of the nine sites (Table AII-4).

*Amino acid composition of harvested seeds*
Soybean is considered a good protein source, but compared to other plant ingredients it contains a lower level of the essential amino acid methionine. The measured levels of amino acids, including methionine, were well within the values reported in the literature, with the exception of tyrosine, where the levels were 10% lower in both A2704-12 and the conventional control compared to the literature values (Table AII-5). The results of the by-site analysis are shown in Table AII-6. At maximum four of nine sites, statistically significant differences in amino acid levels were observed ($p<0.05$). However, the applicant has not provided specific data regarding the concentration differences of essential and/or limiting amino acids between sites.

*Fatty acid composition of harvested seeds*
Soybean oil belongs to the oleic (C18:1) and linoleic (C18:2) rich seed oils. Other main fatty acids in soybean oil are palmitic acid (C16:0), stearic acid (C18:0) and linolenic acid (C18:3). Both linoleic- and linolenic acids are essential fatty acids for humans and other animals. The over-all site averages are compared in table AII-7. The measured levels are in compliance with the composition tables reported in the literature. The by-site analysis revealed that for a majority of the sites (up to seven of nine sites), there were statistically significant differences between the groups for several fatty acids (Table AII-8). This is probably at least partly due to differences in growth conditions. However, the applicant has not provided specific data regarding the concentration differences of essential fatty acids between sites.

*Minerals and Vitamins in harvested seeds*
The over-all site average values for the analysed minerals and vitamins are presented in Table AII-9. All mean values are within the literature range except for folic acid. The mean level of folic acid 2.04 mg/kg (ppm) for the non-transgenic variety was somewhat lower than the minimum of the literature range (2.49 mg/kg (ppm)). For the two treatments of the

transgenic variety, the level was also low, falling just short of the lowest boundary of the literature values (Table AII-9).

The results of the by-site analysis are shown in Table AII-10. Statistically significant differences in minerals and vitamin levels between sites was found at maximum four of nine sites ($p<0.05$). However, the applicant has not provided specific data regarding the concentration differences of essential minerals or vitamins between sites.

There was large variation in the vitamin E results and EFSA asked for additional information (Table AII-11) (ref: Response to EFSA). The reason might be that the A2704 and A2704-12 varieties are optimally adapted to the environmental conditions in the southern states (Illinois, Iowa and Indiana). The seed samples from sites in Ontario, Minnesota, Wisconsin and Nebraska have lower vitamin E mean values and show a higher variation within sites because of some extreme values.

If, however, the extreme values ("outliers") are excluded from the analysis, and average values for the non-transgenic control group and for the transgenic samples are compiled, the differences at five out of eight sites were less than 10% of the mean values. The mean values over-all sites were 43.35 IU/kg dm for non-transgenic seeds and 42.52 IU/kg dm for the transgenic seeds (Table AII-11), which is in good compliance with the literature range (24,9 – 55,1 IU/kg dm) (Tables AII-9 and AII-11).

The applicant argues that environmental conditions at the different sites have a strong impact on the vitamin E content in soybean seed samples as for other vitamins, minerals and bio-active compounds like the isoflavones. Generally, storage conditions may also have an impact on the endogenous content of vitamin E (Turchini, 2010). Due to the variation within and between sites, detection of treatment-site interaction was not possible for vitamin E.

*Antinutrients in harvested seeds*

Most of the mean anti-nutrient values of the non-transgenic and transgenic varieties fall within their respective literature ranges (Table AII-12). Differences exist between reported lectin values. The lectins were measured by two different laboratories, each using a different analytical method. The applicant claims that there is reason to believe that the soybean variety tested, A2704, is a variety with a very low lectin content. Moreover, direct comparison of lectin analytical results is difficult because of the variability that can occur in the analytical methodology (different testing parameters in the hemagglutination test) (Technical dossier). In the by site analysis, statistically significant differences ($p<0.05$) between treatments were observed at six of nine sites for raffinose. For all other antinutrients, the maximum number of sites with statistically significant differences ($p<0.05$) were four of nine (Table AII-13).

*Isoflavones in harvested seeds*

In general, the measured values for the glucosides and the total amounts for daidzein, genistein and glycitein are within the range reported in the literature (Table AII-14). Variability in the isoflavone content of both A2704-12 soybean and A2704 soybean control

was observed and for the aglycones daidzein and genistein, the measured values far exceeded the literature range. Isoflavone content of soybeans can fluctuate depending on various conditions, including location and genotype. Although there were some statistically significant differences between the GM and non-GM soybean within some locations, this was not observed consistently at each location. In addition the values of isoflavone content were within the ranges reported by the OECD (2001). In Table AII-15, the by-site analysis for isoflavone content are presented. Statistically significant differences between treatments were observed at a maximum of four out of nine sites.

### 3.2.2 Field trials for hay and forage production 1996

*Proximate and fiber composition of forage*
The results for the proximate analysis of soybean forage over all three sites are given in Table AII-16. No results for a compound in the transgenic samples exceeded the 20% range built from the control values. The difference in crude protein and fat content between transgenic and control samples are caused mainly by higher values of both fat and crude protein in the non-transgenic soybean than in A2704-12 at the Nebraska site.
The measured values for moisture and crude protein exceed the literature reference values, whereas total carbohydrates and ADF are lower than the standard values. For moisture, the values in the literature are from dried samples, while the values for A2704 are for fresh samples. The high protein content (49% higher than in literature values) may be explained by incomplete separation of the seeds from forage material.

*Proximate and fiber composition of hay*
The content of crude protein, crude fat, ash, ADF and NDF over all sites were all well within the values reported in the literature. The level of total carbohydrates was around 10% lower in both A 2704-12 and the corresponding control compared to the literature values. In the single site comparison, no result for a compound in the transgenic samples exceeded the 20% range built from the control values.

Since soybeans are not grown in Norway, forage and hay material as feed ingredients have only very limited relevance.

## 3.3 Agronomic traits and GM phenotype

The application EFSA/GMO/NL/2005/18, covering authorisation of soybean A2704-12 for all food and feed uses, include data on agronomic and morphological characteristics from field trials in North America during the period 1995-2002. According the applicant, the comparative field observations of plants derived from event A2704-12 and the corresponding near-isogenic cultivar A2704 were made as part of the event evaluation process, and the characteristics evaluated included primarily those important for varietal registration.

*Field trials USA (1995-1997)*

Between 1995 and 1997, a number of field studies were conducted on soybeans derived from event 2704-12, primarily to evaluate efficacy of glufosinate-ammonium. Moreover, quantitative and qualitative observations were also made on agronomic and plant health characteristics. According to the applicant, no significant differences were noted in comparisons between soybeans derived from event A2704-12 and the conventional control (Van Wert, 1998 -Technical Dossier).

The applicant also provided some information on agronomic performance and phenotypic characteristics of soybean varieties derived from event A2704-12 and the near-isogenic control A2704 at three locations during the 1996 growing season. The characteristics that were analysed in this study were plant height, yield, maturity and lodging. The results are summarized in Table AI-2. No results from the statistical analyses of the field trial were, however, available for the VKM GMO Panel.

*Field trials Canada (1998)*
In 1998, field studies were conducted at three Southern Ontario locations to compare the agronomic characteristics between the conventional soybean line A2704 and the transgenic soybean line A2704-12 (Cromar, 1998- Technical Dossier). At each field trial site, soybean A2704-12 and the conventional counterpart were planted following a randomised complete block design with three replicates per site. The plants were grown under conditions typical of commercial production practices. None of the plots were sprayed with glufosinate ammonium. No commercially available soybean varieties were included in the field trials. According to the updated EFSA guidance on risk assessment of food and feed from genetically modified plants (EFSA 2011a), there should be at least three appropriate non-GM reference varieties of the crop that have a known history of safe use at each site. The test of equivalence is used to verify whether the agronomic, phenotypic and compositional characteristics of the GM plant fall within the normal range of natural variation. Such a range of natural variation is estimated from a set of non-GM reference varieties with a history of safe use (EFSA, 2011b) and therefore allows comparisons of the GM plant with a similar food or feed produced without the help of genetic modification and for which there is a well-established history of safe use. These requirements were, however, not in place at the time of submission.

Plant height, yield, protein and oil content were examined at maturity. According to the applicant, poor yield was observed at one location for both the transgenic and non-transgenic varieties due to drought-like conditions. However, both the transgenic and non-transgenic varieties, responded similarly to the stress. No statistically significant differences were seen in the mean values of evaluated parameters of the transgenic line when compared to the non-transgenic line, either within locations or across all locations ($p>0.05$). Table AI-3 summarizes the mean values for plant height, yield, protein and oil content of each line averaged across all locations.

*Field trial USA (2002)*
The applicant also provided data from a field trial conducted at one site in Iowa in 2002 (Shillito, 2003- Technical Dossier). Samples evaluated included soybeans derived from event A2704-12 sprayed twice with glufosinate ammonium and not sprayed with glufosinate ammonium, and soybeans from the conventional counterpart (A2704). No commercially available reference varieties were included in the field trial.

The plots were evaluated for several agronomic characteristics including emergence, stand count, plant vigor and health, flowering date, plant height, days to maturity, yield and seed weight. An unpaired two tailed Student's T-test assuming equal variances was used to test for differences between pairs of treatments for stand counts and plant heights. Table AI-4 summarizes the results of these observations. Based on observations from one field site, the applicant concluded that the growth and development of soybeans derived from the transformation event A2704-12 were similar to the non-transgenic A2704 comparator. A2704-12 showed a very slight difference in maturity dates, and a slightly higher (<105%) yield compared to the conventional control. Stand counts of the soybeans were slightly higher for the transgenic but probably caused by variations in planting density of the trial. The seeds obtained from the transgenic plants showed a slightly lower (92-93%) per seed weight. Plant height was 107-110% of the comparator line when measured at the first flowering, and vigor was similar.

## 3.4 Conclusion

The VKM GMO Panel has considered the available literature on compositional data and found that except for intermittent variations, no biologically relevant differences exist between soybean A2704-12 and its corresponding control A2704 in the analyses of seeds and various processed food and feed commodities. The observed statistical differences between A2704-12 and A2704 are likely to reflect the natural variability, of environmental influences and/or storage conditions on the analyte levels. The data presented do not show unintended effects as a result of the genetic modification.

Based on current knowledge, the VKM GMO Panel concludes that with the exception of the introduced trait, soybean A2704-12 is compositionally, agronomically and morphologically equivalent to its conventional counterpart and other conventional soybean varieties.

# 4 Food and feed safety assessment

## 4.1 Previous evaluation by the VKM GMO Panel

In an earlier risk assessment of soybean A2704-12, the VKM GMO Panel concluded that the soybean A2704-12 is nutritionally equivalent to, and as safe as, conventional non-GM soybean varieties (VKM, 2006).

## 4.2 Product description and intended uses

Soybean A2704-12 was first cultivated in the USA and Canada in 1996, and subsequently cultivated in Argentina in 2011, Brazil in 2010, Canada in 1999, Japan in 2006 and Uruguay in 2012. Soybean A2704-12 was commercialised as food and/or feed in Argentina (2011), Australia (2004, food), Brazil (2010), Canada (2000), China (2010), Colombia (2012, feed), EU (2008), India (2014), Japan (2001 food, 2003 feed), Malaysia (2012), Mexico (2003, food), New Zealand (2004, food), Philippines (2009), Russian Federation (food 2008, feed 2007), Singapore (2014, food), South Africa (2001), South Korea (2009), Taiwan (food 2007), Thailand (food 2013), Turkey (feed 2011), Uruguay (cultivation 2012).

Soybean A2704-12 has been used in food and feed since 1998. According to the applicant the commercial experience since 1998 has confirmed that the production and processing of A2704-12 does not differ from the production and processing of the equivalent foods and feeds, originating from traditional soybean. 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, meals and protein concentrates/isolates. Soybean protein concentrate is a commonly used feed ingredient in Norwegian salmon feeds (www.mattilsynet.no). Since 2008, the Food Safety Authority has given four fish feed producers in Norway extended exemption from seeking approval for inclusion of GM products in fish feeds. The exemption applies to processed, non-viable feed products from 19 different GM varieties. In October 2014, this exemption was not extended.

Soybean A2704-12 has been used in food and feed since 1998. According to the applicant the commercial experience since 1998 has confirmed that the production and processing of A2704-12 does not differ from the production and processing of the equivalent foods and feeds, originating from traditional soybean.

Unprocessed soybeans are not suitable for food and their use in animal feed remains limited because they contain anti-nutritional factors such as saponins, trypsin inhibitors and lectins (OECD 2012). Adequate heat processing inactivates most of the biological activities of antinutritionalthese factors. The main soybean product fed to animals is the defatted/toasted soybean meal. However, aspirated grain fractions, forage, hay, hulls, seed, and silage are also used as feed to a limited extent, primarily to for cattle (OECD 2012). Adequate heat

processing inactivates most of the biological activities of antinutritional factors. Whole soybeans are utilized to produce food products such as soy sprouts, baked soybeans, roasted soybeans, full fat soy flour and the traditional Asian soy foods (miso, soy milk, soy sauce, and tofu) (OECD, 2012). The steps used for processing of soybean produces intended for food and feed 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 before use in food, animal feed or industrial products in the same way as other commercial soybean. 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 A2704-12 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.

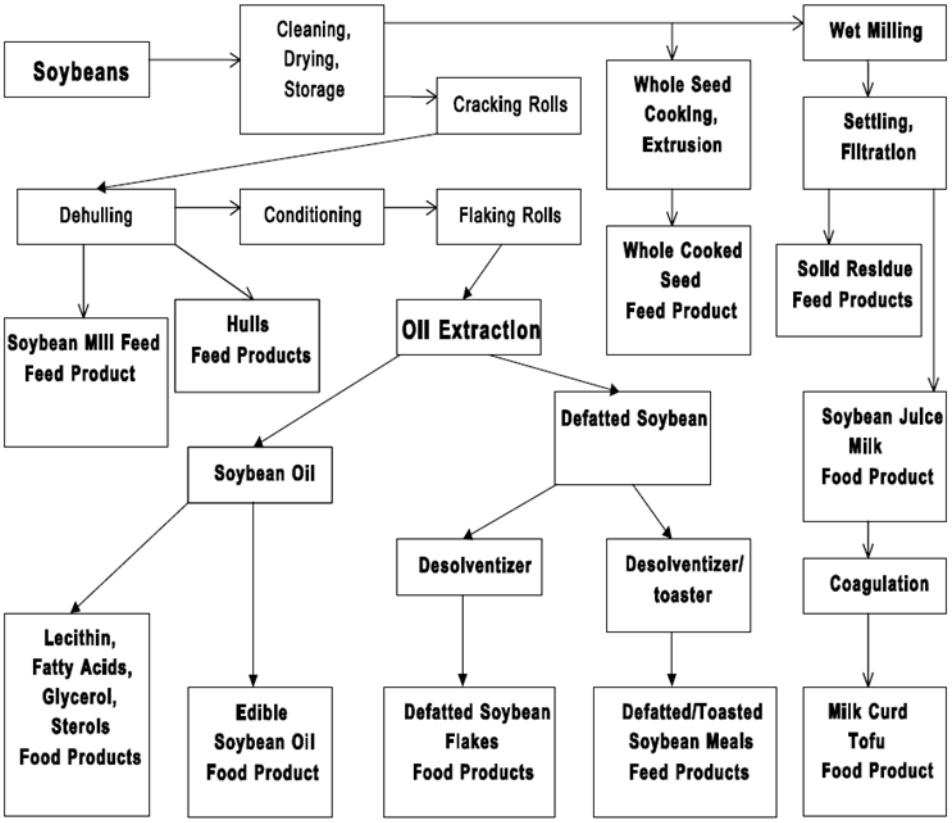


Figure 4.2-1. Processing of soybean, adapted from Waggle and Kolar, 1997, Technical dossier

## 4.3 Effects of processing

The processing steps used to produce the various soy products are shown in figure 4.2-1, above. The first step in processing most soybeans is to separate the oil, either by solvent extraction or by expelling. For this, soybeans are first cracked and de-hulled, then heated to approximately 60 degrees, ground to flakes using 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 can lead to denaturation and loss of protein function. Heat stability study performed by the applicant (ref. Eisdail 2002c; Rascle 2009 (soybean A5547-127)) showed that the PAT-protein was heat stable when incubated up to 30 minutes at 90 °C , and slightly degraded when incubated 60 minutes at 90 °C.

## 4.4 Toxicological assessment of soybean A2704-12

The potential toxicity of genetically modified soybean A2704-12 has been studied in mice, rats and broiler chickens. The studies have utilised various formulations of soybean A2704-12, such as purified PAT protein from *E.coli*, protein concentrate from soybean A2704-12 or whole GM food/feed. Protein concentrate is about 70% soy protein and is basically defatted soy flour without the water-soluble carbohydrates and ethanol-soluble antinutritional factors, and is widely used in formulated feeds for salmonid fishes in Norway. Isolated soy proteins are obtained by extracting the soluble proteins with water at pH 8-9, followed by precipitation at pH 4.5, centrifugation, washing, redispersing and drying. Concentrates and isolates are widely used as functional or nutritional ingredients in a wide variety of food products, mainly in baked foods, breakfast cereals, and some meat products.

In addition to the safety testing conducted by the applicant, a safety testing programme has been conducted on soybean A2704-12 within the Russian Federation, summarised in Tutelyan (2013). The available English transcript describes the program as compliant with the Russian 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 however been difficult to assess for the VKM GMO panel, since this information is only available in Russian. Still, the testing conducted in the Russian Federation is deemed valuable for the risk assessment of soybean A2704-12. 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 in Norwegian fish feed formulations. A brief summary is presented in Appendix IV.

Submitted data demonstrated a low expression of the PAT protein in soybean A2704-12 (approx. 0.6 µg PAT/g fresh weight whole seed; approx. 5 µg/g crude protein in seeds, approx. 9.5 µg/g in toasted meal). The protein was not detectable in soybean oil (applicant dossier). The PAT-protein showed no amino acid sequence homology to known toxic proteins (Hérouet et al 2005). Also, in vitro digestion studies using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) demonstrated that PAT is rapidly degraded under conditions mimicking the stomach and intestine (Esdaile 2002a,b, 2004; Applicant documents). In bovine rumen (paunch) fluid (pH 7.11), total degradation of PAT-protein was observed after 30 min (Schultz, 1993, Applicant document). Digestion of PAT-protein in ground leaves from transgenic zoysiagrass (Z. japonica Steud.) and of PAT protein in total soluble proteins extracted from zoysiagrass has also been confirmed in studies using SGF digestion assays (Sun et al 2010).

### 4.4.1 Acute toxicity testing

An acute intravenous study with the PAT protein has been performed by the applicant to assess potential toxicity in mice (Kennel, 2003; Hérouet). The PAT protein was produced in *E. coli,* purified (>90 %), and administered i.v. to mice in a single dose. The OECD TG 420

fixed dose guidance document (OECD 2001) was used as a basis for assessing the potential acute toxicity. Three groups of mice (each 5 females) were intravenously injected with 1 or 10 mg per kg body weight with either PAT protein, aprotinin or melittin, respectively. All animals were observed for clinical signs daily for 15 days after dosing. Microscopic examination of internal organs was carried out at necropsy. No treatment-related adverse effects were observed in mice administered the PAT protein at the highest dose tested, i.e. 10 mg/kg bw.

The results showed that mice treated i.v. with PAT protein or apoprotein at 10 mg/kg body weight showed no signs of systemic toxicity, whereas all mice treated with melittin at the same dose died within 5 minutes.

Previously, the PAT protein has been assessed by VKM in other genetically modified glufosinate tolerant crop varieties including soybean (A5547-127), with the same conclusion as above. The applicant has not justified the i.v. route of administration or the doses applied.

Acute toxicity testing following i.v. application of the newly expressed proteins is of little additional value to the risk assessment of the repeated human and animal consumption of food and feed derived from GM plants and is therefore not taken into account in this risk assessment. EFSA now discourages the use of acute toxicity studies in risk assessments of GMOs (EFSA, 2011).

### 4.4.2 Repeated dose toxicity testing

#### *4.4.2.1 Two studies of four weeks duration with processed and unprocessed soybean A2704-12, respectively.*

The potential toxicity of the PAT-protein has been assessed in a repeated dose toxicity study (feeding trial) in rats (Pfister et al., 1999).

The applicant provided a 14-day repeated dose feeding study in which groups of 5 Wistar rats (HanIbm:WIST) of each sex were given a low protein diet containing 0, 0.5 or 5.0% (w/w) (group 1, 2 and 3, respectively) of a lyophilised powder of the PAT protein (>98% purity). Group 1 were fed standard rat diet, group 4 were fed non-GM soybean protein. The highest dietary inclusion resulted in a daily dose of ca. 7.6 and 7.9 g/kg body weight for males and females, respectively. The total protein levels in the diets for the control and low-dose groups were adjusted with soya protein from commercial non-GM soybeans to reach a level comparable to that in the diet for the high-dose group. An additional group was fed a standard rodent diet. There were no mortalities, and no relevant influence on food consumption and body weight development induced by the treatments. According to the applicant there were no remarkable findings apart from statistically significant increases in blood cholesterol levels (males of groups 2 (0.5% PAT), 3 (5% PAT) and group 4 (without PAT)) and phospholipid levels (females of group 3 and males of groups 2 and 3).

At the end of the treatment period haematology, clinical chemistry and urine analysis were performed, organ weights were determined, and macroscopic and histopathology examinations of selected organs and tissues were carried out.

This repeated dose toxicity study in rats gave no indications of adverse effects attributable to the PAT protein up to the highest dose tested, which was 5% in the diet, corresponding to 7.9 g/kg bw.

The VKM GMO-Panel notes that this repeated dose feeding study is performed with only 14 days of exposure contrary to the recommended 28 days.

### 4.4.3 Studies on Allergenicity

#### *4.4.3.1 Assessment of allergenicity of the newly expressed protein*

The applicant has assessed the allergenic potential of the PAT protein by bioinformatic comparison of the amino acid sequence of the PAT protein produced in A2704-12 with known database sequences of IgE-dependent allergens, as well as the evaluation of the stability of the protein in an *in vitro* gastric digestion model.

The *pat* gene originates from *Streptomyces viridochromogenes*, a soil microrganism that is not known to be allergenic. The bioinformatic analyses were conducted to assess the potential for allergenicity of the PAT protein sequence (Hérouet, 2004b, applicant dossier). The total amino acid sequence of PAT was compared to epitopes of known IgE-dependent allergens. In addition a search was performed of potential N-glycosylation sites with known consensus sequences that may be found in IgE-dependent allergenic proteins. A search was also performed on all protein sequences presented in reference databases, i.e. IgE-dependent allergen, gliadin and glutenin sequences database (AD4) assembled from publicly available databases (GenBank, EMBL, PIR, NRL3D version of RCSB PDB and SwissProt) and from current literature.

The applicant has also carried out amino acid sequence homology study of the complete amino sequence of PAT with protein sequences of known toxin and allergens in the databases NRL-3D, PIR, DAD, GenPept, Uniprot_TrEMBL and Uniprot-SwissProt.

The amino acid sequence of the PAT 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 PAT amino acid sequence was also screened against the allergen database with an algorithm that scans for a window of eight linearly contiguous amino acids. Such identities might indicate the presence of potentially cross-reactive allergenic epitopes. The results of this bioinformatics search indicate that the PAT 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 proteins associated with coeliac disease.

European and Asian patients allergic to soybean and/or other foods do not express IgE that specifically bind the purified PAT protein (Chang et al., 2003; Batista et al., 2005; Kim et al., 2006a, 2006b; Hoff et al., 2007). The purified PAT protein 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 produced PAT protein in A2704-12 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 IgE-dependent allergens in A2704-12 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 A2704-12 to A5403, a traditional non-GM soybean with the same genetic background as A2704-12, and to three commercially available traditional soybean varieties. The analysis of the protein extracts prepared from A2704-12 indicate 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 non-GM soybean varieties, demonstrating that the production of the PAT protein in A2704-12 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 A2704-12. Namely, that none of the individuals undergoing allergenicity tests reacted differently to A2704-12 than to traditional soybean samples (Batista et al., 2005). Moreover, a lack of detectable allergenicity towards the produced PAT protein was reported (Batista et al., 2005; Chang et al., 2003).

Allergenicity of the soybean could be changed 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 A2704-12 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. Using 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 A2704-12 compared to 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 A2704-12 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 PAT and known allergens including the “Derf 2” mite allergen with sera of patients allergic to certain foods and mites.

#### *4.4.3.3 Assessment of allergenicity of proteins derived 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 (with the exception of the introduced traits; see 3.2 and 3.3) and no reported difference in allergenic potential of the whole plant (see 4.4.3.2) have been identified, no increased IgE-mediated allergenicity is anticipated for soybean A2704-12.

### 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 risk of allergic reactions. 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 suggest 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 (Ref Adjuvansrapport?). Previously it was assumed that the epithelial cells of the intestine were permanently held together tightly by the so-called tight junctions. More recent knowledge shows that these complex protein structures are dynamic and can become less tightly joined, i.e. more “leaky”, 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 reactions), 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 lead to allergic sensitisation (Brandtzaeg & Tolo, 1977; Lim & Rowley, 1982).

The PAT-protein has not been reported to have adjuvant properties.

## 4.5 Nutritional assessment of GM food/feed

Compositional analyses of soybean A2704-12 indicate nutritional equivalence to the non-GM control soybean with comparable genetic background and to the published range of values in the literature (see 3.2). The nutritional equivalence between soybean and non-GM control soybean has been further shown by the results from feeding studies with broiler chickens (see chapter 4.5.2.1).

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, the non-GM comparator treated with conventional herbicide management regimes, and the GM plant treated with the conventional herbicide management regimes. The broiler chicken study provided by the applicant is 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 both with and without 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.

### 4.5.1 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 A2704-12, the estimated average exposure of the European consumer to products of soybean A2704-12 would be approximately 3.4-3.7 g/person/ day (Technical dossier).

In Table 4.5.1-1 the mean intake of soy protein/day for an adult person 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 where milk products were replaced with soy products was composed. Both menus are included in appendix III.

**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 supply 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 A2704-12 meal in the EU would be 21% for broiler chickens, 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 protein concentrate (SPC) is one important protein source in salmon feeds (Directorate of Fisheries, Biomass statistics 2013). The average inclusion level of SPC in feed for Atlantic salmon is 25%, total SPC used for fish feed production in 2013 was calculated to be approximately 375 000 tons (Annual Sustainability report, Skretting, 2013).

Assuming that 100% of the SPC was derived from soybean A2704-12, the estimated average exposure of Atlantic salmon (post smolt, 200 g) to products of soybean A2704-12would 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 GMOs (*e.g.* below 0.9%) (Spilsberg et al 2014). Samples of all imported SPCs are analysed for the presence of five transgene sequences commonly found in GMOs. These five DNA specific targets are: the 35S promoter (p35S), *Agrobacterium* nopalin synthase terminator (tNOS), *ctp2-cp4epsps*, the *bar* gene from *Streptomyces hygroscopicus,* and the *pat* gene from *Streptomyces viridichromogenes*. The methodology is highly sensitive and capable of detecting minute amounts of GM-material. Additional analyses may also be carried out to determine the specific GMOs present in a sample.

### 4.5.2 Nutritional assessment of feed derived from the GM plant

#### *4.5.2.1 Applicant's data for nutritional assessment*

*Broiler feeding study*

The applicant provided a 15-day broiler feeding study using Ross 208 broilers, instead of the generally accepted 42-day broiler feeding study for fattening (ILSI, 2003). Only a starter diet containing 18.24% soybean meal in the feed was used. Two groups were compared, one was fed glufosinate tolerant soybean A2704-12 and the control group was fed the parental soybean 2704 (Leeson et al., 2001). Seventy two male and seventy two female broilers were obtained at one day of age. The birds were weighed and allocated (by sex) at random to one of the two treatment groups, replicated six times with six birds per replicate. The broilers were identified by wing-bands before randomly assigned to a pen and treatment group.

The soybean meal was heat treated at a core temperature of 115 °C to inactivate trypsin inhibitor. Birds were fed only starter diets for 15 days. Feed and water were provided ad libitum during the test. The birds received coccidiostats in the diet and the growth promoter STAFAC.

Group mean body weights for the birds were measure at day 0, and prior to euthanasia at the end of the test period (Day 15). Birds were observed twice daily. Each diet was prepared for the starter period and were based on commonly used corn-soybean containing diets. The treatments varied only in the source of soybeans used in each diet.

At the end of the study there were no differences (t-test procedure) between groups of broilers fed soybean A2704-12 meal diet compared with broilers fed conventional soybean event 2704 in terms of body weight, feed intake, feed conversion efficiency and weight gain. Also, there were no significant differences between the mortality of broiler fed A2704-12 compared with the parental soybean event 2704 for male, female or combined male and female broilers. Mortality data were analysed by Fisher's exact test.

The VKM GMO panel is of the opinion that the study is short relative to the ILSI (2003) recommended 42 day broiler feeding studies for fattening. However, because the broilers had a weight gain of 7-8 times initial body weight during the 15-day study period, it is deemed of value as a nutritional study for young broilers during rapid growth.

## 4.6 Conclusion

A 14-day repeated dose toxicity study with rats fed PAT protein, as well as a nutritional assessment trial with broilers fed diets containing soybean A2704-12 did not indicate any adverse effects. The PAT protein in A2704-12 does not show sequence resemblance to known toxins or IgE-dependent allergens, nor has it been reported to cause IgE-mediated allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that soybean A2704-12 is nutritionally equivalent to and as safe as its conventional counterpart and other conventional soybean varieties.

# 5 Environmental risk assessment

Considering the scope of the application EFSA/GMO/NL/2005/18, the environmental risk assessment is concerned with the accidental release into the environment of viable soybean A2704-12 seeds during transport and/or processing, and with indirect exposure to microorganisms in the gastrointestinal tract and soil/water, mainly via ingestion by animals, their intestinal contents and faeces .

## 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 conditions.

There is no reason to assume that expression of the introduced characteristics in soybean A2704-12 will increase the potential to establish feral populations. A series of field trials with soybean A2704-12 was conducted by the applicant at several locations in the USA (1996) and Canada (1998) to compare the agronomic performance and field characteristics of soybean A2704-12 with its comparators (see section 3.3). With the exception of targeted responses to the presence of glufosinate herbicides, the agronomic and morphological field trial data did not show major changes in plant characteristics indicating altered fitness, persistence and invasiveness of soybean A2704-12 plants compared to its conventional counterpart.

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 A2704-12, or changes to its survivability (including over-wintering), persistence or invasive capacity. Because the general characteristics of soybean A2704-12 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 A2704-12 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 A2704-12. 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 A2704-12 to unrelated species such as bacteria.

It has, however, been 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 compared to 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). Following oral intake, it has been shown that DNA from GM soybean 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 A2704-12 will transfer to and establish itself 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 *pat* gene from A2704-12 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, while these 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 A2704-12 will not present a risk of spread of transgenes to organic or conventionally grown varieties, wild populations or closely related species in Norway.

## 5.3 Interactions between the GM plant and target organisms

Considering the intended uses of soybean A2704-12, 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 A2704-12, excluding cultivation, potential interactions of the GM soybean 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 A2704-12, 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 A2704-12, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure to microorganisms in the gastrointestinal tract and soil/water, mainly via intestinal content and faeces from animals fed feeds containing soybean A2704-12.

Soybean A2704-12 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 A2704-12. 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 is 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 A2704-12 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 A2704-12.

# 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 two full length functional copies of the *pat* gene are present in the soybean A2704-12 genome. Similarity searches in 2006, with databases of known toxins and allergens did not indicate any potential for production of harmful proteins or polypeptides as a result of the genetic modification. Southern blot and segregation analyses show that the introduced gene elements are stably inherited and expressed over several generations, and consistent with the observed phenotypic characteristics of soybean A2704-12. The VKM GMO Panel concludes that the molecular characterisation of soybean A2704-12 does not indicate a safety concern.

**Comparative assessments**

The VKM GMO Panel has considered the available literature on compositional data and found that except for intermittent variations, no biologically relevant differences exist between soybean A2704-12 and its corresponding control A2704 in the analyses of seeds and various processed food and feed commodities. The observed statistical differences between A2704-12 and A2704 are likely to reflect the natural variability, of environmental influences and/or storage conditions on the analyte levels. The data presented do not show unintended effects as a result of the genetic modification.

Based on current knowledge, the VKM GMO Panel concludes that with the exception of the introduced trait, soybean A2704-12 is compositionally, agronomically and morphologically equivalent to its conventional counterpart and other conventional soybean varieties.

**Food and feed risk assessment**

A 14-day repeated dose toxicity study with rats fed PAT protein, as well as a nutritional assessment trial with broilers fed diets containing soybean A2704-12 did not indicate any adverse effects. The PAT protein in A2704-12 does not show sequence resemblance to known toxins or IgE-dependent allergens, nor has it been reported to cause IgE-mediated allergic reactions.

Based on current knowledge, the VKM GMO Panel concludes that soybean A2704-12 is nutritionally equivalent to and as safe as its conventional counterpart and other conventional soybean varieties.

### Environmental assessment

Considering the intended uses of soybean A2704-12, excluding cultivation, the environmental risk assessment is concerned with accidental release into the environment of viable grains during transportation and processing, and indirect exposure to microorganisms in the gastrointestinal tract and soil/water, mainly via intestinal content and faeces from animals fed feeds containing soybean A2704-12.

Soybean A2704-12 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 A2704-12. 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 is 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 and considering the intended usage, the VKM GMO Panel concludes that soybean A2704-12 is as safe as its conventional counterpart and commercial soybean varieties. With the exception of the introduced trait, soybean A2704-12 is nutritionally, morphologically and agronomically equivalent to conventional soybean varieties.

Likewise, the VKM GMO Panel concludes that soybean A2704-12 does not represent a discernible environmental risk in Norway.

# 8 Data gaps

Herbicide tolerant (HT) crops permit the use of broad-spectrum herbicides such as glufosinate-ammonium 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 of 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 glufosinate-ammonium 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

**Table AI-1**: Summary of molecular studies performed by the applicant, reported in the original submission (Table 6. In Technical dossier)

| Type of analysis | Parameters analyzed | Comparator | Appendix Ref. | Relevance in Chapter D |
|---|---|---|---|---|
| Southern blot hybridization | Copy number, Vector backbone sequences | A2704 | De Beuckeleer & Botterman, 1999 | 2(a); 2(b); 6(a) |
| | Insert stability in multiple generations | A2704 | De Beuckeleer, 1998 | 2 (c); 5 |
| | Insert stability in different locations | A2704 | Currier, 2005 | 2 (c); 5 |
| | Insert stability in different genetic backgrounds | Several 2704-12 lines; A2704 | Currier, 2005 | 2(c); 5 |
| | Flanking sequence determination | A2704 | De Beuckeleer, 2002 | 2(d); 7.8 |
| Polymerase Chain Reaction | Sequence information | ----- | Berghman & De Beuckeleer, 2002b | 2; 2(b); 2(d); 2(e); |
| | Flanking sequence determination | A2704 | De Beuckeleer, 2002 | 2(d); 7.8 |
| BLAST sequence similarity research | Integration site | A2704 | De Beuckeleer & Botterman, 1999 | 2(c); 2(d); 7.8 |
| | Similarities between flanking sequences and known genes | Genomic database | De Beuckeleer, 2003 | 2(d); 7.8 |
| | Open Reading Frame research | Genomic database | Berghman, 2005 Hérouet, 2005 | 3(c); 7.8 |
| Northern blot hybridization | Cryptic expression analysis | A2704; *bla* RNA | De Beuckeleer & Botterman 1997 | 3(b); 7.8 |
| ELISA[a] | PAT expression analysis | A2704 | Scott & Currier, 2003 Shillito, 1997, 2001, 2003b; Barraj, 1998; Cromar, 1999; Currier, 2003 | 3(a) 3(b) |

[a] Enzyme Linked ImmunoSorbent Assay

**Table AI-2.** Agronomic characteristics evaluated in GM soybean A2704-12 and conventional control (A2704) in field trials in the USA in 1996.

| Plant[b)] | Yield (bushels/acre) | Maturity (date) | Height (inches) | Lodging (1-5) |
|---|---|---|---|---|
| A2704 | 61.6 | Sept. 28.5 | 35.5 | 1.4 |
| A2704-12 lot 1 | 61.9 | Sept. 30.5 | 31.8 | 1.7 |
| A2704-12 lot 2 | 60.5 | Sept. 29.5 | 32.2 | 1.4 |
| Std. Error | 1.2 | 0.28 | 0.62 | 0.26 |

a) Agronomic data collected from 3 sites. Values are the average of all rows of A2704 (parent line) and the average of all rows from 2 seed lots of A2704-12.

b) Std. Error = Standard Error of Means. Yield was calculated for each plot based on weight of seed at harvest, row length and number of rows. Days to maturity is an objective evaluation of the date at which half the plants in each plot reach maturity. Height is the measured height of several plants within each plot that are representative of the plot as a whole. Lodging is a subjective rating of the plot as a whole.

**Table AI-3** Summary of agronomic Traits (height, yield, protein and oil) between non-transgenic (A2704) and transgenic (A2704-12) soybean lines[1)], averaged across locations.

| Variable | A2704 | A2704-12 | P-value[3] |
|---|---|---|---|
| Height (cm) | 79.44 ± 16.09 | 81.22 ± 17.22 | 0.824 |
| Yield (kg/ha) | 2842 ± 1608 | 2919 ± 1717 | 0.922 |
| Protein % | 44.27 ± 1.37 | 44.17 ± 1.28 | 0.875 |
| Oil % | 22.16 ± 1.04 | 21.79 ± 1.03 | 0.464 |

**Table AI-4** Comparison of soybean agronomic characteristics A2704 parent line and Event A2704-12 Lines, unsprayed with glufosinate ammonium and sprayed with glufosinate ammonium.

| Characteristic | A2704 (Parent Line) | A2704-12 Unsprayed | A2704-12 Sprayed |
|---|---|---|---|
| Date of emergence[1)] | May 21, 2002 | May 21, 2002 | May 21, 2002 |
| Stand count[2)] | $75.5^{a}$ ± 4.6 | $82.9^{b}$ ± 3.7 | $81.5^{b}$ ± 4.3 |
| Plant vigor[3)] May 21 | 9 | 9 | 9 |
| Plant vigor[3)] June 4 | 8 | 9 | 9 |
| Plant health[4)] July 4 | 5 | 5 | 5 |
| Time to flowering | 50 days | 54 days | 54 days |
| Plant height (inches)[5)] | $15.9^{a}$ ± 1.8 | $17.2^{ab}$ ± 1.3 | $17.6^{b}$ ± 1.8 |
| Days to maturity | 138 | 140 | 142 |
| Yield - bushels/acre (% moisture) | 43.0 (11.2%) | 45.96 (10.6%) | 44.09 (10.7%) |
| Yield- kg/ha (% moisture) | 2892 (11.2%) | 3091 (10.6%) | 2965 (10.7%) |
| 100 Seed weight – grams [5) 6)] | $14.15^{a}$ ± 0.46 | $13.06^{b}$ ± 0.24 | $13.21^{b}$ ± 0.51 |
| 100 Seed weight – grams [5) 7)] | $16.42^{a}$ ± 0.53 | $16.02^{ab}$ ± 0.30 | $16.05^{b}$ ± 0.62 |

c) Date at which 50% of the crop had emerged

d)Mean and standard deviation are presented. Count represents plants per 10 feet of row at the V2 stage. Values with the same superscript were not different (p>0.05) by the student's t-test. A2704 may have been planted slightly thin because of the moisture on the seed coming out of cold storage.

e) 9 rating = excellent emergence, very healthy looking plants 8 rating = good emergence, leaf(ves) dimpled

4) 5 rating = No injury/effect seen

5) Values with the same superscript were not different (p> 0.05) by the student's t-test

6) Not corrected for moisture

7) Corrected to 13% moisture

# Appendix II

**Table AII-1.** Compounds Analysed in the Raw Agriculture Commodities of Soybean and processed Soybean Products

| Compound | Seed | Hay | Forage | Hulls | Meal | Toasted meal | Protein Isolate | Refined oil | Crude lecithin |
|---|---|---|---|---|---|---|---|---|---|
| Proximates# | X | X | X | X | X | X | X## | | |
| Ca, P, K, Mg, Na, Fe | X | | | | | | | | |
| Vitamin B1, B2, E, Folic acid | X | | | | | | | | |
| Amino Acids | X | | | | X | X | X | | |
| Fatty Acids | X | | | | | | | X | |
| Stachyose | X | | | | | | | | |
| Raffinose | X | | | | | | | | |
| Phytic acid | X | | | | X | X | | | |
| Trypsin Inhibitors | X | | | | X | X | | | |
| Lectins | X | | | | X | X | | | |
| Isoflavones | X | | | | X | X | | | |
| Phosphatides | | | | | | | | | X |

\# Proximates comprise moisture, crude protein, crude fat, ash, total carbohydrates (calculated), acid detergent fibre and neutral detergent fibre.

## Soy protein isolate was only analysed for moisture and crude protein content.

**Table AII-2.** Sources of Standard Composition Data (from Technical dossier)

| Source | Abbreviation |
|---|---|
| Belitz, Grosch (1985); Lehrbuch der Lebensmittelchemie; 2nd ed.; Springer Verlag; Berlin, pp 559-572. | BG |
| CRC (1983) Handbook of Processing and Utilization in Agriculture, Volume 2, Part 2 - Plant Products. pp 27-32, 39, 41-45, 50, 57-59, 83-89. | CRC |
| Douglas J.S., (1996), Recommended Compositional and nutritional parameters to test in soybean, TAS Inc. Washington, USA | TAS |
| Ensminger M.E., et al. (1990) Feeds and Nutrition, 2nd edition, Ensminger Publishing Co. | Ensminger |
| Feed Industry Red Book 1995, Reference and Buyers' Guide. Communications Marketing, Inc., Eden Prairie, Minnesota. | Red Book |
| Hui Y.H., (1992), Encyclopedia of food science and technology, p2389-2396, Vol4, John Wiley & sons Inc. New York | Hui |
| Kakade M.L. et al., (1972), Biochemical and nutritional assessment of different varieties of soybeans. J. Agr. Food Chem., p. 87-90, 20 | Kakade |
| Kellems, R.O., Church, D.C. (1998); Livestock Feeds and Feeding; 4th ed.; Prentice Hall; New Jersey. | Kellems |
| Macgregor C.A. (1994); Directory of Feeds & Feed Ingredients; 2nd ed.; W.D. Hoard & Sons Company, Fort Atkinson, Wiskonsin, USA | Macgregor |
| Nasner, A. (1985), Die antioxidativen Eigenschaften von Lecithin, Fette, Seifen Anstrichmittel, p477-481, Jg. 87, Nr. 12, | Nasner |
| North Carolina Feed Report (1994-1995) Bulletin of the NC Dept. Agriculture, Raleigh, NC. Number 261. | NCDA |
| Nutrient Requirements of Beef Cattle, (1984) 6th edition, National Academy Press, Washington D.C. | Nut Beef |
| Nutrient Requirements of Dairy Cattle, (1978) 5th edition, National Academy Press, Washington D.C. | Nut Cow |
| OECD. 2001. Consensus Document on Compositional Considerations for New Varieties of Soybean: Key Food and Feed Nutrients and Anti-nutrients. ENV/JM/MONO(2001)15. | OECD |
| Pardun H., (1989), Pflanzenlecithine- wertvolle Hilfs- und Wirkstoffe?, Fat Sci Technol. P45-58, Jg. 91, Nr. 2 | Pardun |
| Scherz H. and Senser F.; (1994); Food Composition and Nutrition Tables; 5th ed.; CRC Press Boca Raton; Florida; USA. | FSK |
| Sotirhos N. *et al.*, High Performance Liquid Chromatographic Analysis of Soybean Phospholipids, (1986), Fette, Seifen Anstrichmittel Jg. 88, Nr. 1 | Sotirhos |
| United States – Canadian Tables of Feed Composition, (1982) 3rd Revision National Academy Press, Washington D.C. | USCA |
| U.S. Department of Agriculture, Agricultural Research Service. USDA Nutrient Database for Standard Reference, Release 13, Nutrient Data Laboratory, Home Page, http://www.nal.usda.gov/fnic/foodcomp accessed May 15, 2001. | USDA |
| U.S. Department of Agriculture, Agricultural Research Service. USDA-IOWA State University Database on the Isoflavone content of Foods, Release 1.2-2000, Home Page, http://www.nal.usda.gov/fnic/foodcomp accessed May 15, 2001. | USDA-IOWA |

**Table AII-3.** Mean values and standard deviations for the content of proximates over-all sites, listed separately for the three treatments: nontransgenic A2704, transgenic A 2407-12 not sprayed, transgenic A 2407-12 sprayed with glufosinate. Standard values from the literature is included (figure adapted from Öberdoerfer, 2003, Technical dossier).

| Parameter | Non-Transgenic (Mean ± SD) | | | Transgenic Not sprayed (Mean ± SD) | | | Transgenic Sprayed (Mean ± SD) | | | Standard-Values [a] |
|---|---|---|---|---|---|---|---|---|---|---|
| Moisture %fw | 9.64 | ± | 2.61 | 10.51 | ± | 3.19 | 10.25 | ± | 2.79 | 5,6 - 12,0 |
| Crude Protein %dm | 41.58 | ± | 2.24 | 41.89 | ± | 1.75 | 41.92 | ± | 1.63 | 32,0 - 43,6 |
| Crude Fat %dm | 20.87 | ± | 1.31 | 20.74 | ± | 1.06 | 20.68 | ± | 1.10 | 15,5 - 24,7 |
| Ash %dm | 5.17 | ± | 0.40 | 5.12 | ± | 0.34 | 5.11 | ± | 0.30 | 4,2 - 6,4 |
| Total carbo-hydrates %dm [b] | 32.51 | ± | 1.89 | 32.25 | ± | 1.65 | 32.29 | ± | 1.85 | 31,7 - 38,9 |
| ADF %dm | **6.54** | ± | 0.85 | **6.90** | ± | 1.35 | **6.96** | ± | 1.39 | 9,0 - 11,3 |
| NDF %dm | 9.26 | ± | 1.12 | 9.72 | ± | 1.46 | 9.74 | ± | 0.93 | 5,0 - 14,9 |

[a] Standard values from table 2 of appendix A.

**Table AII-4.** Summary of the by site analysis of the proximate data for the A; nontransgenic A2704, B; transgenic A 2407-12 not sprayed, C; transgenic A 2407-12 sprayed with glufosinate. (adapted from Rattemeyer, 2006).

| Summary t-test procedures *) | A vs. B | | A vs. C | |
|---|---|---|---|---|
| | significant | not significant | significant | not significant |
| Moisture | 3 | 6 | 2 | 7 |
| Crude Fat | 3 | 6 | 3 | 6 |
| Crude Protein | 2 | 7 | 6 | 3 |
| Ash | 3 | 6 | 1 | 8 |
| Acid detergent fibre | 2 | 7 | 3 | 6 |
| Neutral detergent fibre | - | 9 | - | 9 |
| Total Carbohydrates | 1 | 8 | 3 | 6 |

*) N of sites with significant ($p < 0.05$) and not significant ($p \geq 0.05$) treatment differences.

**Table AII-5.** Mean levels and standard deviations for Amino Acids in Seeds of nontransgenic A2704, transgenic A 2407-12 not sprayed, transgenic A 2407-12 sprayed with glufosinate, over-all sites. (Figure adapted from Öberdoerfer, 2003, Technical dossier).

| Parameter | %dm | | |
|---|---|---|---|
| | Non-Transgenic (Mean ± SD) | Transgenic Not sprayed (Mean ± SD) | Transgenic Sprayed (Mean ± SD) | Standard-Values [a] |
| Alanine | 1.68 ± 0.13 | 1.68 ± 0.13 | 1.68 ± 0.15 | 1,67 - 1,88 |
| Arginine | 2.96 ± 0.35 | 3.04 ± 0.33 | 2.98 ± 0.27 | 2,17 - 3,11 |
| Aspartic acid | 4.85 ± 0.40 | 4.78 ± 0.42 | 4.84 ± 0.32 | 4,36 - 5,01 |
| Cystine | 0.60 ± 0.05 | 0.61 ± 0.06 | 0.61 ± 0.06 | 0,45 - 0,73 |
| Glutamic acid | 7.36 ± 0.61 | 7.38 ± 0.50 | 7.32 ± 0.50 | 7,09 - 7,72 |
| Glycine | 1.81 ± 0.21 | 1.78 ± 0.20 | 1.81 ± 0.22 | 1,55 - 2,27 |
| Histidine | 1.04 ± 0.06 | 1.05 ± 0.06 | 1.05 ± 0.06 | 0,84 - 1,22 |
| Isoleucine | 1.82 ± 0.12 | 1.85 ± 0.11 | 1.83 ± 0.10 | 1,71 - 2,32 |
| Leucine | 3.00 ± 0.20 | 3.03 ± 0.16 | 3.02 ± 0.15 | 2,2 - 4,0 |
| Lysine | 2.54 ± 0.14 | 2.56 ± 0.11 | 2.56 ± 0.11 | 1,55 - 2,67 |
| Methionine | 0.56 ± 0.02 | 0.57 ± 0.02 | 0.57 ± 0.03 | 0,50 - 0,76 |
| Phenylalanine | 1.95 ± 0.15 | 2.05 ± 0.41 | 1.96 ± 0.11 | 1,60 - 2,39 |
| Proline | 2.09 ± 0.18 | 2.11 ± 0.19 | 2.08 ± 0.15 | 1,99 - 2,33 |
| Serine | 2.05 ± 0.16 | 2.02 ± 0.14 | 2.06 ± 0.13 | 1,85 - 2,39 |
| Threonine | 1.65 ± 0.10 | 1.63 ± 0.10 | 1.65 ± 0.08 | 1,40 - 1,89 |
| Tryptophan | 0.58 ± 0.05 | 0.57 ± 0.05 | 0.58 ± 0.05 | 0,43 - 0,67 |
| Tyrosine | **1.19** ± 0.09 | **1.20** ± 0.08 | **1.19** ± 0.08 | 1,28 - 1,51 |
| Valine | 1.93 ± 0.13 | 1.96 ± 0.11 | 1.93 ± 0.10 | 1,50 - 2,44 |

[a] Standard values from table 4 of appendix A.

**Table AII-6**. Summary of the by site analysis of Amino Acids (adapted from Rattemeyer, 2006).

| Summary t-test procedures *) | A vs. B | | A vs. C | |
|---|---|---|---|---|
| | significant | not significant | significant | not significant |
| Alanine | 1 | 8 | 2 | 7 |
| Arginine | 3 | 6 | 3 | 6 |
| Aspartic Acid | 3 | 6 | 4 | 5 |
| Cystine | 2 | 7 | 1 | 8 |
| Glutamic Acid | 2 | 7 | 4 | 5 |
| Glycine | 1 | 8 | 1 | 8 |
| Histidine | 3 | 6 | 4 | 5 |
| Isoleucine | 4 | 5 | 4 | 5 |
| Leucine | 3 | 6 | 4 | 5 |
| Lysine | 4 | 5 | 4 | 5 |
| Methionine | - | 9 | 3 | 6 |
| Phenylalanine | 3 | 6 | 4 | 5 |
| Proline | 2 | 7 | 1 | 8 |
| Serine | 3 | 6 | 2 | 7 |
| Threonine | 2 | 7 | 1 | 8 |
| Tryptophan | 3 | 6 | 4 | 5 |
| Tyrosine | 2 | 7 | 3 | 6 |
| Valine | 3 | 6 | 3 | 6 |

*) N of sites with significant ($p < 0.05$) and not significant ($p \geq 0.05$) treatment differences

**Table AII-7**. Mean levels and standard deviations for Fatty Acids in Seeds of nontransgenic A2704, transgenic A 2407-12 not sprayed, transgenic A 2407-12 sprayed with glufosinate, over-all sites. (Figure adapted from Öberdoerfer, 2003, Technical dossier).

| Parameter | % Relative | | | |
|---|---|---|---|---|
| | Non-Transgenic (Mean ± SD) | Transgenic Not sprayed (Mean ± SD) | Transgenic Sprayed (Mean ± SD) | Standard-Values [a] |
| *Saturated* | | | | |
| Palmitic C16:0 | 9.36 ± 0.46 | 9.22 ± 0.50 | 9.18 ± 0.42 | 7 - 12 |
| Margaric C17:0 | <0,10 – 0,12 [b] | <0,10 - 0,12 [b] | <0,10 - 0,12 [b] | - |
| Stearic C18:0 | 4.53 ± 0.39 | 4.67 ± 0.49 | 4.73 ± 0.54 | 2 - 5 |
| Arachidic C20:0 | 0.35 ± 0.02 | 0.36 ± 0.03 | 0.37 ± 0.04 | <1,0 |
| Behenic C22:0 | 0.42 ± 0.08 | 0.46 ± 0.12 | 0.45 ± 0.09 | - |
| Lignoceric C24:0 | 0.12 ± 0.01 | 0.13 ± 0.01 | 0.13 ± 0.01 | - |
| *Total Saturated* | 14,89 | 14,95 | 14,98 | 9 - 17 |
| *Mono-unsaturated* | | | | |
| Palmitoleic C16:1 | <0,10 – 0,16 [b] | <0,10 – 0,16 [b] | <0,10 – 0,15 [b] | 0 - 0,48 |
| Oleic C18:1 | 22.04 ± 1.41 | 23.05 ± 1.88 | 22.97 ± 1.99 | 19 - 34 |
| Gadoleic C20:1 | 0.20 ± 0.04 | 0.21 ± 0.04 | 0.21 ± 0.05 | 0,17 |
| Erucic C22:1 | <0,10 – 0,47 [b] | <0,10 – 0,66 [b] | <0,10 – 0,58 [b] | - |
| *Total Mono uns.* | 22,60 | 23,75 | 23,64 | 19,3 - 34,7 |
| *Poly-unsaturated* | | | | |
| Linoleic C18:2 | 53.84 ± 1.09 | 52.90 ± 1.20 | 52.93 ± 1.43 | 48 - 60 |
| Linolenic C18:3 | 8.50 ± 0.87 | 8.24 ± 1.28 | 8.32 ± 1.10 | 2 - 10 |
| *Total Poly uns.* | 62,34 | 61,14 | 61,13 | 50 - 70 |
| *Grand Total* | 99,83 | 99,84 | 99,75 | - |

[a] Standard values from table 5 of appendix A

**Table AII-8.** Summary of the by site analysis of Fatty Acids in seeds. (from Rattemeyer, 2006).

| Summary t-test procedures *) | A vs. B | | A vs. C | |
|---|---|---|---|---|
| | significant | not significant | significant | not significant |
| C16:0 Hexadecanoic | 4 | 5 | 5 | 4 |
| C16:1 Hexadecenoic # | 2 | 7 | 2 | 7 |
| C17:0 Heptadecanoic # | 1 | 8 | 1 | 7 |
| C18:0 Octadecanoic | 6 | 3 | 5 | 4 |
| C18:1 Octadecenoic | 7 | 2 | 6 | 3 |
| C18:2 Octadecadienoic | 7 | 2 | 6 | 3 |
| C18:3 Octadecatrienoic | 5 | 4 | 4 | 5 |
| C20:0 Eicosanoic | 5 | 4 | 6 | 3 |
| C20:1 Eicosenoic | 1 | 8 | - | 9 |
| C22:0 Docosanoic | 3 | 6 | 5 | 4 |
| C22:1 Docosenoic # | 3 | 5 | 1 | 8 |
| C24:0 Tetracosanoic # | 4 | 4 | 3 | 8 |

*) N of sites with significant ($p < 0.05$) and not significant ($p \geq 0.05$) treatment differencences
#) 'not significant' was also assumed if all samples of a site were equal or below the limit of detection for the respective two treatments

**Table AII-9.** Mean levels and standard deviations for minerals and vitamins in Seeds of nontransgenic A2704, transgenic A 2407-12 not sprayed, transgenic A 2407-12 sprayed with glufosinate, over-all sites. (figure adapted from Öberdoerfer, 2003, Technical dossier).

| Parameter | On dry matter basis | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Non-Transgenic (Mean ± SD) | | | Transgenic Not sprayed (Mean ± SD) | | | Transgenic Sprayed (Mean ± SD) | | | Standard-Values [a] |
| Calcium % | 0.27 | ± | 0.05 | 0.26 | ± | 0.05 | 0.27 | ± | 0.06 | 0,21 - 0,34 |
| Phosphorus % | 0.64 | ± | 0.08 | 0.64 | ± | 0.09 | 0.64 | ± | 0.08 | 0,49 - 0,77 |
| Potassium % | 1.94 | ± | 0.17 | 1.86 | ± | 0.24 | 1.94 | ± | 0.17 | 1,4 - 2,1 |
| Magnesium % | 0.26 | ± | 0.03 | 0.27 | ± | 0.03 | 0.27 | ± | 0.03 | 0,21 - 0,32 |
| Sodium % | 0.02 | ± | 0.01 | 0.02 | ± | 0.01 | 0.03 | ± | 0.01 | 0,0022 - 0,02 |
| Iron ppm | 78.91 | ± | 11.80 | 89.37 | ± | 22.44 | 82.71 | ± | 15.62 | 71,0 - 172 |
| Vitamin B1 ppm | 9.76 | ± | 2.20 | 8.99 | ± | 1.21 | 8.90 | ± | 1.43 | 3,24 - 16,02 |
| Vitamin B2 ppm | 5.09 | ± | 1.33 | 5.22 | ± | 1.01 | 4.92 | ± | 0.93 | 2,7 - 14,5 |
| Folic acid ppm | **2.04** | **±** | **0.70** | **2.44** | ± | 0.79 | **2.40** | ± | 0.80 | 2,49 - 4,10 |
| Vitamin E IU/kg | 43.35 | ± | 67.98 | 55.45 | ± | 68.47 | 29.61 | ± | 20.75 | 24,9 - 55,1 |

[a] Standard values from table 3 of appendix A.

**Table AII-10.** Summary of the by site analysis of minerals and vitamins. (adapted from Rattemeyer, 2006).

| Summary t-test procedures *) | A vs. B | | A vs. C | |
|---|---|---|---|---|
| | significant | not significant | significant | not significant |
| Calcium | 4 | 5 | 3 | 6 |
| Phosphorus | 1 | 8 | - | 9 |
| Potassium | 2 | 7 | 2 | 7 |
| Magnesium | 1 | 8 | 1 | 8 |
| Sodium # | 2 | 6 | 2 | 5 |
| Iron | 3 | 6 | 4 | 5 |
| Vitamin B1 | 2 | 7 | 2 | 7 |
| Vitamin B2 | 2 | 7 | 1 | 8 |
| Folic acid | 2 | 7 | 2 | 7 |
| Vitamin E | 1 | 7 | 1 | 7 |

*) N of sites with significant ($p < 0.05$) and not significant ($p \geq 0.05$) treatment differences.
#) 'not significant' was also assumed if all samples of a site were equal or below the limit of detection for the respective two treatments

**Table AII-11**. Vitamin E content (IU/kg) of seed of soybean event A2704-12 (not sprayed and sprayed with Liberty (glufosinate)) and the non-transgenic counterpart obtained from different field trial locations (Technical dossier, Response to EFSA)

| Site | Non-transgenic seeds | Transgenic seeds | Transgenic seeds Liberty treated | Difference to control mean (%) |
|---|---|---|---|---|
| Illinois_99 | 40,0 | 47,4 | 26,3 | |
| | 48,1 | 52,8 | 43,7 | |
| | 39,2 | 41,0 | 43,3 | |
| Mean | 42,4 | 42,4 | | 0 |
| Nebraska_99 | 20,0 | 26,1 | 34,0 | |
| | 27,3 | 7,2 | 61,4 | |
| | 29,1 | 7,7 | 23,1 | |
| Mean | 25,5 | 26,6 | | + 4,3 |
| Wisconsin_99 | 21,4 | 22,7 | 6,5 | |
| | 18,9 | 56,8 | 9,0 | |
| | 17,3 | 299 # | 6,5 | |
| Mean | 19,2 | 20,3 | | 5,7 |
| Ontario_99 | 19,7 | 130 # | 37,4 | |
| | 22,5 | 6,5 | 9,5 | |
| | 356 # | 6,5 | 6,5 | |
| Mean | **21,1** | **13,3** | | **37,0** |
| Iowa_00 | 50,6 | 76,9 | 60,2 | |
| | 41,1 | 52,1 | 58,3 | |
| | 41,9 | 58,0 | 70,1 | |
| Mean | **44,5** | **62,6** | | **40,7** |
| Indiana_00 | 50,6 | 42,1 | 35,2 | |
| | 42,2 | 200 # | 48,2 | |
| | 46,9 | 51,9 | 45,0 | |
| Mean | 46,6 | 44,5 | | 4,5 |
| Wisconsin_00 | 9,4 | 76,5 | 6,5 | |
| | 39,1 | 9,2 | 11,6 | |
| | 25,5 | 6,5 | 35,4 | |
| Mean | 24,7 | 24,3 | | 1,6 |
| Minnesota_00 | 11,6 | 6,5 | 12,1 | |
| | 13,1 | 12,5 | 11,2 | |
| | 8,9 | 34,6 | 9,7 | |
| Mean | **11,2** | **14,4** | | **28,6** |
| Ontario_00 | Excluded from analysis since 6 of 9 results < limit of quantification. | | | |
| Over-all sites (excluding Ontario_00) | 43,35 ± 67,98 | 42,52 ± 51,73 | | 1,91 |

# suspicious outlier excluded from calculation

**Table AII-12.** Mean levels and standard deviations for Anti-nutrients in Seeds of nontransgenic A2704, transgenic A 2407-12 not sprayed, transgenic A 2407-12 sprayed with glufosinate, over-all sites. (Figure adapted from Öberdoerfer, 2003, Technical dossier).

| Parameter | On dry matter basis | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Non-Transgenic (Mean ± SD) | | | Transgenic Not sprayed (Mean ± SD) | | | Transgenic Sprayed (Mean ± SD) | | | Standard-Values [a] |
| Stachyose (%dm) | 4.23 | ± | 0.72 | 4.31 | ± | 0.63 | 4.30 | ± | 0.67 | 1,48 - 6,30 |
| Raffinose (%dm) | 0.72 | ± | 0.26 | 0.83 | ± | 0.24 | 0.74 | ± | 0.20 | 0,11 - 1,28 |
| Phytic acid (%dm) | 1.67 | ± | 0.21 | 1.67 | ± | 0.20 | 1.67 | ± | 0.19 | 1,00 - 2,74 |
| Trypsin inhibition TIU/g dm | 62.81 | ± | 12.16 | 66.93 | ± | 14.35 | 69.07 | ± | 14.01 | 40,0 - 73,6 |
| Lectin HU/mg dm (Ralston) | **7.70** | ± | 3.89 | **6.05** | ± | 4.27 | **5.69** | ± | 3.75 | 14,8 - 129 |
| Lectin HU/mg dm (Covance) | **1.17** | ± | 0.90 | **0.73** | ± | 0.61 | **1.15** | ± | 1.24 | 14,8 -129 |

[a] Standard values from table 6 of appendix A

**Table AII-13.** Summary of the by site analysis of Anti-nutrients (from Rattemeyer, 2006).

| Summary t-test procedures *) | A vs. B | | A vs. C | |
|---|---|---|---|---|
| | significant | not significant | significant | not significant |
| Stachyose | 3 | 6 | 2 | 7 |
| Raffinose | 6 | 3 | 3 | 6 |
| Phytic acid | 3 | 6 | 1 | 8 |
| Trypsin inhibition | 2 | 7 | 4 | 5 |
| Lectin (Ralston) | 1 | 5 | 3 | 3 |
| Lectin (Covance) | - | 5 | - | 5 |

*) N of sites with significant ($p < 0.05$) and not significant ($p \geq 0.05$) treatment differences.

**Table AII-14.** Mean levels and standard deviations for Isoflavones in Seeds of nontransgenic A2704, transgenic A 2407-12 not sprayed, transgenic A 2407-12 sprayed with glufosinate, over-all sites. (Figure adapted from Öberdoerfer, 2003, Technical dossier).

| Parameter | Ppm dm | | | | | | | | | Standard-Values [a] |
|---|---|---|---|---|---|---|---|---|---|---|
| | Non-Transgenic (Mean ± SD) | | | Transgenic Not sprayed (Mean ± SD) | | | Transgenic Sprayed (Mean ± SD) | | | |
| Daidzin | 343.31 | ± | 209.97 | 248.56 | ± | 176.72 | 242.96 | ± | 160.22 | 13 - 1244 [b] |
| Daidzein | **149.42** | **±** | **180.47** | **340.44** | **±** | **320.17** | **263.00** | **±** | **265.41** | 5 - 35 |
| Total Daidzein | 1261.58 | ± | 420.87 | 1154.70 | ± | 375.46 | 1093.15 | ± | 419.90 | 202 - 2060 |
| Genistin | 345.08 | ± | 204.62 | 265.44 | ± | 185.96 | 265.63 | ± | 176.52 | 16 - 2105 [b] |
| Genistein | **100.54** | **±** | **126.51** | **229.74** | **±** | **255.81** | **173.19** | **±** | **183.49** | 0,3 - 46 |
| Total Genistein | 1316.38 | ± | 460.06 | 1149.30 | ± | 514.09 | 1140.37 | ± | 523.09 | 315 - 2680 |
| Glycitin | 84.77 | ± | 39.15 | 71.56 | ± | 39.85 | 67.63 | ± | 37.97 | 53 - 338 [b] |
| Glycitein | 14.08 | ± | 19.68 | 32.19 | ± | 37.64 | 22.56 | ± | 23.86 | 1 - 80 |
| Total Glycitein | 253.88 | ± | 74.29 | 240.74 | ± | 66.71 | 225.04 | ± | 71.65 | 82 - 1070 |
| Total Isoflavones | 2831.62 | ± | 922.30 | 2545.93 | ± | 919.75 | 2459.74 | ± | 976.05 | 440 - 9100 [b] |

[a] Standard values from table 7 of appendix A

**Table AII-15**. Summary of the by site analysis of Isoflavones (from Rattemeyer, 2006).

| Summary t-test procedures *) | A vs. B | | A vs. C | |
|---|---|---|---|---|
| | significant | not significant | significant | not significant |
| Daidzin | 3 | 6 | 4 | 5 |
| Daidzein | 3 | 6 | 2 | 7 |
| Total Daidzein Compounds | 3 | 6 | 3 | 6 |
| Genistin | 2 | 7 | 3 | 6 |
| Genistein | 2 | 7 | 3 | 6 |
| Total Genistein Compounds | 2 | 7 | 3 | 6 |
| Glycitin | - | 9 | 1 | 8 |
| Glycitein | 1 | 7 | 1 | 7 |
| Total Glycitein Compounds | 1 | 8 | - | 9 |
| Total Isoflavones | 3 | 6 | 3 | 6 |

*) N of sites with significant ($p < 0.05$) and not significant ($p \geq 0.05$) treatment differences.

**Table AII-16.** Proximatesa in Soybean Forage of A2704-12 and the non-transgenic A2704, compared to commercial soybean varieties (Figure adapted from Öberdoerfer, 2003, Technical dossier).

| Parameter | % Dry matter | | |
|---|---|---|---|
| | Non-transgenic (Mean ± SD) | Transgenic Not sprayed (Mean ± SD) | Standard Values[b] |
| Moisture %fw | 80,08 ± 1,68 | 80,71 ± 2,32 | 10,0 - 79,0 |
| Crude protein | 26,48 ± 4,90 | 23,47 ± 1,97 | 11,2 - 17,7 |
| Crude fat | 3,16 ± 0,77 | 2,58 ± 0,40 | 2,7 - 5,1 |
| Ash | 10,21 ± 1,94 | 9,80 ± 0,27 | 8,8 - 10,5 |
| Total carbohydrates [c] | 58,83 ± 13,46 | 57,14 ± 8,98 | 67,1 - 76,9 |
| ADF | 30,48 ± 5,69 | 30,68 ± 2,76 | 32,0 - 40,0 |
| NDF | 38,07 ± 8,18 | 37,69 ± 4,18 | 34 - 40 |

NF Data not found
[a] Mean and standard deviation over-all-sites taken from table 4 of appendix E
[b] Standard values from table 9 in appendix A.
[c] Total carbohydrates calculated as 100% - (crude protein %dm + crude fat %dm + ash %dm)

# Appendix III

## 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 IV

A rather extensive safety testing programme has been conducted on soybean A2704-12 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:

### Assessment of allergenicity of proteins derived from the GM plant

Allergenicity of the soybean could be changed 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.

Assessment of possible sensibilisation of A2704-12 on the immune response to endogenous metabolic products was carried out by testing sensitivity to histamine in mice (Tutelyan 2013). 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. The authors concluded that histamine did not affect behavior or mortality of the mice and that soybean A2704-12 does not contain sensitising ingredient for mice.

### Studies on Immunotoxicity

#### *Potential effect on humoral component of immune system*

*Level of hemagglutination after injecting sheep erythrocytes*

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 mouse lines C57Bl/6 (low sensitivity to SE) and CBA (high sensitivity to SE) (Tutelyan, 2013). 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 A2704-12, 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. In CBA mice fed diet with soy protein concentrate derived from transgenic

soybean line A2704-12 or from conventional soybean, the antibody titer was 1:20 starting from post-immunization Day 14. Under the same conditions, the antibodies appeared in C57Bl/6 mice on post-immunization Day 14 (1:16–1:64) and they could be detected on Day 21 after immunization (1:32). The synthesis rate of the antibodies raised against SE in C57Bl/6 and CBA mice lines fed diet with soy protein concentrate derived from transgenic soybean and in mice of the same lines fed on conventional soy protein concentrate was similar. The investigators concluded that soybean line A2704-12 produces no effect on the humoral component of the immune system compared to control.

*Hypersensitivity reaction to sheep erythrocytes (SE)*

The possible immunomodulating effect of transgenic soybean was assessed with delayed hypersensitivity reaction to sheep erythrocytes (SE) (Tutelyan, 2013). C57Bl/6 - and CBA mice were used in this test. Each strain was divided in three groups, one group was fed soybean protein concentrate from A2704-12, one was fed conventional soybean protein concentrate and one was fed without soybean (control group). 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. According to the Russian investigators the synthesis rate of the antibodies raised against SE in C57Bl/6 and CBA mice lines fed diet with soy protein concentrate derived from transgenic soybean and in mice of the same lines fed on conventional soy protein concentrate was similar.

*Effect of soybean A2704-12 on susceptibility to Salmonella typhimurium*

Effect of soybean A2704-12 on susceptibility to *Salmonella typhimurium* was investigated in mice (Tutelyan, 2013). 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 100 to $10^5$ microbial cells per mouse and varied on a 10-fold basis. The post-injection observation period was 21 days. The lifetime of the mice in the test group and the control mice were 15.4 and 16.1 days, correspondingly. $LD_{50}$ values of control and test mice were 154 and 76 microbial cells per mouse, respectively 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 control group took longer to die than the test group, although the differences in LD50 values remained within the experimental error. Thus, introduction of protein concentrate derived from transgenic soybean line A2704-12 into mouse diet produced no effect on the humoral and cellular components of the immune system, did not sensitize the mouse organism, and did not disturb the natural resistance against typical infection such as murine typhus. According to

the authors, these data support the conclusion that transgenic soybean line A2704-12 has no immunomodulating properties.

*Assessment of systemic anaphylaxis*

The potential impact of soybean A2704-12 on systemic anaphylaxis was investigated in rats (Tutelyan, 2013). 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 = 46) weighing 140 ± 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 A2704-12 for 28 days. During the entire experiment the rats of both groups fed diet with protein concentrate derived from conventional and transgenic soybean line A2704–12 grew normally.

On experimental days 1, 3 and 5, the rats were sensitized 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 $IgG_1$ and $IgG_4$ 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 A2704-12 were insignificant ($p>0.05$). There was only an insignificant increase of anaphylactic reaction and mortality in the A2704-12 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 A2704-12 demonstrated a declining trend in comparison with the control group. The degree of sensitization 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 A2704-12 did not elevate allergic reactivity and sensitization towards the model allergen in test rats in comparison with the control rats fed conventional soybean.

### *Potential genotoxicity of soybean A2704-12*

The potential genotoxicity of soybean A2704-12 was investigated in an *in vivo* genotoxicity experiment in mice (Tutelyan, 2013). The study was conducted in accordance with the requirements of the Ministry of Health of the Russian Federation authorized for risk and safety assessment of food derived from GM sources (MUK 2.3.2.970-00, 2008) The genotoxicity studies were carried out on male C57Bl/6 and female CBA mice sensitive to mutagenesis. For 45 days, the mice weighing 16–18 g were fed diet with protein concentrate from soybean A2704-12 (test group n=15 male mice) or its conventional counterpart (control group n=12 male mice) 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 45-day feeding period, the C57Bl/6 test (n=15) and C57Bl/6 control (n=12) 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 test mice had no overt chromosomal abnormalities. Only single segments and the gaps were detected, and their number did not surpass 2% (the level of spontaneous mutation characteristic of this species). These chromosomal aberrations are not preserved in mitosis and are eliminated during the following divisions of cell nucleus.

To examine the dominant lethal mutations in gametes, 60 test females were dissected to count and analyze 332 embryos and 363 corpus lutea. The pre-implantation mortality was approximately equal in the control and test groups.

At the stages of early and late spermatids or mature, the post-implantation mortality in the test group (the most reliable index of mutagenic activity of the examined substance) was smaller in test group then in the control group.

There was no induced mortality at these stages of early and late spermatids or mature spermatozoa, indicating absence of the negative effect on spermiogenesis in mice fed protein concentrate derived from transgenic soybean line A2704-12.

The Russian investigators concluded that glufosinate-tolerant soybean line A2704-12 produced no mutagenic effect in the described experiments.

**Toxicological assessment of the whole GM food / feed**

### *Subchronic feeding studies in rats with soy protein concentrate derived from soybean A2704-12*

A feeding study over 180 days with soy protein concentrate was conducted on male Wistar rats. Biochemical, hematological, and morphological parameters were monitored in accordance with the requirements of the Ministry of Health of the Russian Federation authorized for risk and safety assessment of food derived from GM sources (Tutelyan, 2013). Male Wistar rats (n = 60) with a body weight of 85-95 g were randomised into two groups. The test rats were provided daily with protein concentrate derived from the transgenic soybean line A2407-12. The control rats were provided with the same amount of protein concentrate prepared from the conventional non-GM counterpart. The amount of soybean protein concentrate in the semi-synthetic rat diet was 22.5 g per 100 g diet. Samples were collected on days 30 and 180 of the experiment.

During the entire duration of the experiment, the general condition of the rats was similar in the control and test groups. No mortality was observed in either group. 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 180. Hematological assays showed that feeding rats with protein concentrate derived from transgenic soybean line A2704-12 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 180 days.