

**13 December 2016**

**[31–16]**

**Supporting document 1**

Safety Assessment Report (at Approval)– Application A1128

Food derived from reduced Acrylamide Potential & Browning Potato Line E12

# Summary and conclusions

## Background

A genetically modified (GM) potato line with OECD Unique Identifier SPS-ØØE12-8 (herein referred to as E12) has been developed by SPS International Inc. The potato has been modified such that the raw tubers show less browning when they are bruised, cut or damaged (referred to as blackspot bruising) and the tubers have reduced potential to produce acrylamide when cooked at high temperatures.

This potato has been genetically modified using a RNA interference (RNAi) approach. Gene fragments from four genes were introduced into E12 and were intended to supress the expression of four endogenous potato genes. The introduced DNA fragments are derived from potato (*Solanum tuberosum*)and a related species (*Solanum verrucosum*). No other genetic modification has been introduced and no new proteins are produced in line E12.

The four potato genes targeted for reduced expression in the tubers were: *asparagine synthetase-1* (*Asn1*), *phosphorylase-L* (*PhL*), *water dikinase R1* (*R1*), and *polyphenol oxidase-5* (*Ppo5*). The aim of the suppression of *Asn1* was to reduce levels of free asparagine. The aim of suppression of *PhL* and *R1* was to reduce levels of the reducing sugars, fructose and glucose. Collectively, the reduction of free asparagine and reducing sugars was expected to result in potato tubers with reduced acrylamide potential. Reduced expression of *Ppo5* was expected to result in tubers with reduced blackspot bruising.

In conducting a safety assessment of food derived from E12, a number of criteria have been addressed including: a characterisation of the transferred gene sequences, their origin, function and stability in the potato genome; the changes at the level of DNA, RNA and protein in the whole food; compositional analyses; and evaluation of intended and unintended changes.

This safety assessment report addresses only food safety and nutritional issues of the GM food *per se*. It therefore does not address:

* environmental risks related to the environmental release of GM plants used in food production
* the safety of animal feed, or animals fed with feed, derived from GM plants
* the safety of food derived from the non-GM (conventional) plant.

## History of use

On a global scale, potato is the fourth most important food crop following maize, rice and wheat and is cultivated in over 100 countries. It has been cultivated for human consumption for thousands of years and has a long history of safe use as human food. Potatoes are typically cooked before consumption and are processed into food commodities such as potato crisps, pre-cooked French fries, potato flour and potato starch. Potato is also used as a feed for domestic livestock and for the production of alcohol.

## Molecular characterisation

E12 was generated through *Agrobacterium*-mediated transformation with a single T-DNA containing two expression cassettes. The cassettes contain gene fragments that when transcribed were expected to lead to the suppression of four endogenous potato genes*: Asn1*, *PhL*, *R1*, and *Ppo5*. Comprehensive molecular analyses of E12 indicate there is a single insertion site containing a single complete copy of the T-DNA insert. The introduced genetic elements are stably maintained from one generation to the next. There are no antibiotic resistance marker genes present in the line and no plasmid backbone has been incorporated into the transgenic locus.

Northern blot analyses were used to compare the mRNA levels associated with the four endogenous potato genes particularly in tubers of E12. These analyses demonstrated a reduction in the expression levels of *Asn1* and *Ppo5,* but not *PhL* and *R1.*

Consistent with the above, additional analyses showed the following: a) compositional analysis confirmed a significantly lower asparagine level in E12 than the control; b) a colorimetric assay showed that in E12 tubers, compared to tubers of the control, there was significantly lower activity of polyphenol oxidase (PPO), the enzyme that leads to a darkening of damaged tissue; and c) the fructose and glucose levels in E12 tubers did not consistently differ significantly from those in the non-GM control tubers. Despite the expression of *PhL* and *R1* not being reduced, the reduction in expression of *Asn1* was sufficient by itself to produce the desired trait (reduced acrylamide production) in cooked products (fries) of E12.

## Compositional analyses

Detailed compositional analyses were done to establish the nutritional adequacy of tubers from E12 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, vitamins, minerals, total amino acids, free amino acids, sucrose, reducing sugars (fructose and glucose), and anti-nutrients. The levels were compared to levels in: a) the non-GM potato parental line, Russet Burbank; b) a reference range compiled from results taken for Russet Burbank and eight other non-GM commercial varieties grown under the same or similar conditions; and c) levels recorded in the literature. Only seven of the 38 analytes considered deviated from the control in a statistically significant manner; two of these differences (free asparagine and free glutamine) were expected due to the genetic modification. However, the mean levels of both of these analytes fell within the reference range, and all but two of the remaining significantly different analytes fell within the combined literature range. The two analytes (asparagine + aspartic acid and cysteine) were both lower than the combined literature range but were not considered biologically significant. Additionally, for all analytes showing a significant difference, the difference between the mean of E12 and the control was smaller than the variation within the control.

It was therefore concluded that tubers from E12 are compositionally equivalent to tubers from conventional potato varieties.

## Conclusion

No potential public health and safety concerns have been identified in the assessment of E12. On the basis of the data provided in the present Application, and other available information, food derived from E12 is considered to be as safe for human consumption as food derived from conventional potato varieties.

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# List of Abbreviations

|  |  |
| --- | --- |
| *Asn1* | *asparagine synthetase-1* gene |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pairs |
| DNA | deoxyribonucleic acid |
| FASTA | Fast Alignment Search Tool - All |
| FSANZ | Food Standards Australia New Zealand |
| fw | fresh weight of tissue |
| g | gram |
| GM | genetically modified |
| kb | kilo base |
| kcal | kilocalorie |
| LB | Left Border of T-DNA (*Agrobacterium tumefaciens*) |
| mg | milligram |
| NCBI | National Centre for Biotechnology Information |
| NBY | nutrient broth-yeast |
| OECD | Organisation for Economic Co-operation and Development |
| ORF | open reading frame |
| *PhL* | *phosphorylase-L* gene |
| *pAgp* | ADP glucose phyrophosphorylase gene promoter |
| *pGbss* | granule-bound starch synthase promoter  |
| ppb | parts per billion |
| PPO | polyphenol oxidase |
| *Ppo5* | *polyphenol oxidase-5 gene* |
| ppm | parts per million |
| PCR | polymerase chain reaction |
| *R1* | *water dikinase R1 gene* |
| RB | Right Border of T-DNA (*Agrobacterium tumefaciens*) |
| PPO | polyphenol oxidase |
| dsRNA | double stranded RNA |
| RNA | ribonucleic acid |
| RNAi | RNA interference |
| mRNA | messenger RNA |
| SAS | Statistical Analysis Software |
| the code | Australia New Zealand Food Standards Code |
| T-DNA | transfer DNA |
| US | United States of America |

# 1 Introduction

SPS International Inc (SPS), a subsidiary of the United States of America (US) food and agribusiness company J.R. Simplot, has submitted an application to FSANZ to vary Schedule 26 in the *Australia New Zealand Food Standards Code* (the Code) to include food from a new genetically modified (GM) potato line E12 with OECD Unique Identifier SPS-ØØE12-8 (herein referred to as E12). This potato has been genetically modified using an approach called RNA interference (RNAi). In this approach, fragments of four genes have been introduced with the intent of suppressing the level of expression of four native potato genes. No other genetic modification has been introduced and no new proteins are produced in line E12.

The suppression of the four genes was expected to have two main effects. The first effect was that less acrylamide would be produced when the potatoes are cooked at high temperature, such as in roasting or deep frying. While many cooked foods contain acrylamide, it is regarded as prudent to not consume too much (FSANZ 2014; FDA 2016). The second effect was a reduction in browning in raw potato tubers when they are bruised, cut or damaged (a phenomenon known as blackspot bruising). Potatoes with blackspot bruising are either trimmed or rejected during processing, resulting in economic loss.

The genetic modification was designed to reduce the expression of *asparagine synthetase-1* (*Asn1*), *phosphorylase-L* (*PhL*), *water dikinase R1* (*R1*), and the *polyphenol oxidase-5* gene (*Ppo5*). The introduced DNA fragments are derived from potato (*Solanum tuberosum*)except for *Ppo5,* which is derived from a related species, *S. verrucosum*. The aim of the suppression of *Asn1* was to reduce free asparagine, and the aim of suppression of *PhL* and *R1* was to lower the content of the reducing sugars, fructose and glucose. Collectively the reduced expression of these three genes was intended to result in potatoes with reduced acrylamide potential. Reduced expression of *Ppo5* was intended to confer a reduced browning phenotype resulting in potatoes with reduced blackspot bruising.

At this point, SPS does not intend to import potato line E12 into Australia or New Zealand or cultivate it in either country. The aim of this application is to obtain food approval for imported processed foods that may contain E12. GM potato line E12 was approved for use as human food and animal feed in the USA (2015) and Canada (2016). Foods derived from GM plants utilising RNAi for the silencing of endogenous plant genes have previously been assessed by FSANZ in soybean (A1018, A1049) and lucerne (A1085).

# 2 History of use

## 2.1 Host organism

Unless otherwise referenced, the following description of the host organism was adapted from biology documents published by the Canadian Food Inspection Agency (CFIA 2015) and the Organisation for Economic Co-operation and Development (OECD 1997); statistical data is from the Food and Agriculture Organization of the United Nations (FAOSTAT3 2015).

Potato (*Solanum tuberosum*) originates from South America where it has been cultivated for human consumption for thousands of years (Ugent and Peterson 1988) and has a long history of safe use as human food. It is a perennial plant but is grown as an annual for commercial production. Potato is propagated vegetatively using small tubers or pieces of tuber typically referred to as seed or seed potatoes. However, vegetative propagation may perpetuate diseases.

Thus, production of seed potato typically follows a certification system which includes starting with disease free stock, isolation from other potato production areas, control of disease-spreading insects and frequent inspection and culling of diseased plants.

Potato can also be propagated via sexually produced seed. However, seed production and breeding are challenging. The species contains both diploid and tetraploid varieties which vary greatly with regard to self- and cross-compatibility. Pollen sterility occurs frequently and ovule sterility occasionally within the species. Many varieties do not produce seed. The degree, duration and response of flowering behaviour to environmental conditions are greatly influenced by the variety. The applicant has stated that the potato variety used for transformation, Russet Burbank, has sparse blossoms which are infertile.

Currently, potato is the fourth most important food crop following maize, rice and wheat and is cultivated in over 100 countries. World potato production was estimated at over 385 million tonnes in 2014 with China the top producer at 96 million tonnes. In 2011, worldwide consumption of fresh and processed potatoes was about 35 kg/person/year with higher consumption in Australia and New Zealand at about 50 kg/person/year.

Potato is not a major crop in Australia or New Zealand; production in 2014 was about 1.2 million tonnes and 440,000 tonnes, respectively. Australia exported about 54,000 tonnes and imported about 110,000 tonnes of potatoes and processed potato products in 2013. In 2013 New Zealand exported about 100,000 tonnes and imported about 15,000 tonnes of potato and processed potato products.

Potatoes are typically cooked before consumption and are processed into food commodities such as potato crisps, pre-cooked French fries, potato flour and potato starch. Potato is also used as a feed for domestic livestock and processed into alcohol. The variety of potato genetically modified, Russet Burbank, was developed in the USA by Luther Burbank in 1914. It is the standard for baking and processing quality and has good long-term storage (Potato Association of America 2016a). Russet Burbank was introduced into Australia in the 1970’s and is Australia’s most sought after variety for processed French-fries (Agriculture Victoria[[1]](#footnote-2)).

Potato is not known to cause disease in humans or animals and has a long history of safe use as food. Potato and other members of the Solanaceae family, such as tomatoes and eggplants, naturally contain glycoalkaloids which are toxic to humans if consumed in high quantities. Humans are rarely exposed to high levels of the toxin. A maximum limit of 20 mg/100 g fresh weight is the widely accepted safety limit for total glycoalkaloids in registered potato varieties. Proper storage conditions and peeling the potato before use help reduce levels of glycoalkaloids. Allergies to potatoes appear to be relatively uncommon.

Exposure to potatoes, whether cooked or raw, may elicit an allergenic response in humans to the major tuber-storage protein patatin (Sol t 1). Four other proteins (Sol t 2, Sol t 3.0101, Sol t 3.0102 and Sol t 4) are related to soybean trypsin inhibitors and may cause reactions in atopic children. Cooking can reduce the allergenicity and this appears to be due to aggregation with other potato proteins rather than denaturation of patatin itself (Shewry 2003; Camire et al. 2009 and references therein).

##

## 2.2 Donor organisms

### 2.2.1 *Solanum tuberosum*

The majority of the introduced DNA sequences are derived from the host species (*S. tuberosum*), variety Ranger Russet, a commercial potato variety released in the USA in 1991 (Potato Association of America 2016b). It is the source of the ADP glucose phyrophosphorylase gene promoter (*pAgp)* and the granule-bound starch synthase promoter (p*Gbss*), the *PhL*, *R1* and *Asn1* gene fragments, Left and Right Border region sequences and the spacer DNA (see Table 1). The intervening DNA sequences were also derived from *S. tuberosum*.

### 2.2.2 *Solanum verrucosum*

The *Ppo5* gene fragments were derived from *S. verrucosum*. It is a wild, edible species of potato from Mexico (Facciola 1998; CFIA 2015) that has been used as a bridging species for the conventional breeding of desirable traits from other wild species of potato into the domesticated potato (*S. tuberosum*) (Jansky and Hamernik 2009).

# 3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

* the transformation method together with a detailed description of the DNA sequences introduced to the host genome
* a characterisation of the inserted DNA including any rearrangements that may have occurred as a consequence of the transformation
* the genetic stability of the inserted DNA and any accompanying expressed traits.

The Applicant has submitted the following studies regarding the molecular characterisation of line E12.

**Studies submitted:**

2015. Molecular Characterization of the DNA Insert in Russet Burbank E12. Report 15-29-SPS-MOL-01. Simplot Plant Sciences. SPS Regulatory Lab (unpublished).

2015. Evidence for the absence of plasmid backbone DNA in Russet Burbank E12. Report 15-52-SPS-MOL-01. Simplot Plant Sciences, SPS Regulatory Lab (unpublished).

2016. Characterization of the Insertion Site in Russet Burbank Event 12. Report 15-57-SPS-MOL-03. Simplot Plant Sciences, SPS Regulatory Lab (unpublished).

2015. Stability of the DNA Insert in Russet Burbank E12. Report 15-64-SPS-MOL-01. Simplot Plant Sciences, SPS Regulatory Lab (unpublished).

2015. RNA expression of down-regulated genes in Russet Burbank E12. Report 15-71-SPS-MOL. SPS Regulatory Lab (unpublished).

2015. Construction of pSIM1278. Report 15-75-SPS-MOL. SPS Regulatory Lab (unpublished).

2015. Efficacy of polyphenol oxidase downregulation in E12 tubers. Report 15-84-SPS-MOL. Simplot Plant Sciences (unpublished).

2016. Allergen and Toxin Evaluation of Open Reading Frames in Russet Burbank E12. Report 16-46-SPS-MOL. Simplot Plant Science, SPS Regulatory Lab. (unpublished).

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## 3.1 Method used in the genetic modification

E12 was genetically modified following a modified version of the methodology of Richael et al. (2008). Internode segments of *in vitro* grown potato plants (variety Russet Burbank) were aseptically excised from 4 week old plants and transformed using a disarmed *Agrobacterium tumefaciens* strain (AGL1) with the T-DNA from plasmid vector pSIM1278 (Figure 1).

After co-culture with the *Agrobacterium* carrying the vector, infected explants were transferred to regeneration medium to promote callus formation. The regeneration medium contained 300 mg/L timentin, an antibacterial agent, to inhibit *Agrobacterium* growth. Developing calli were transferred every four weeks to fresh media until visible shoots developed. Any plantlets containing the pSIM1278 backbone DNA and expressing *ipt* were discarded based on abnormal growth due to overproduction of the plant hormone cytokinin.

Shoots with wild-type phenotype were transferred to rooting media for about three weeks. Leaf samples from plantlets which had formed roots were tested using polymerase chain reaction (PCR) to identify those containing the T-DNA insert from pSIM1278. Growing tips from plantlets containing the T-DNA insert were transferred to fresh media containing timentin to grow new plantlets and to continue to select against *Agrobacterium*. Plantlets went through a second round of PCR-testing and transfer of growing tips to fresh media. Mature plantlets were tested a third time via PCR for the presence of the T-DNA insert.

Leaf samples from PCR-positive plants were also tested for the absence of *Agrobacterium* by incubating sterile leaves in nutrient broth-yeast (NBY) extract broth for 14 days. Plants showing no bacterial growth were tested a second time to confirm the absence of *Agrobacterium*. Tips of plants testing positive for *Agrobacterium* were transferred to fresh rooting medium containing timentin and this process was continued until *Agrobacterium* was confirmed absent using the NBY assay.

Mature plantlets were then transferred to soil in a greenhouse facility. Tubers from these plants (G0 tubers) were tested for suppression of the *Ppo5* gene using the catechtol assay (a colorimetric assay, Section 3.4.4) to select events for further study. Southern blot analysis was used to identify plants with single inserts and these were further assessed for insert integrity and gene suppression, as well as phenotypic and agronomic characteristics under field trial conditions (Section 3.4)



*Figure 1: Genes and regulatory elements contained in plasmid pSIM1278*

## 3.2 Function and regulation of introduced gene fragments

Information on the genetic elements in the T-DNA used for transformation is summarised in Tables 1 and 2. The complete plasmid is 19,660 bp comprising the 9,512 bp backbone and 10,148 bp T-DNA (Figure 1). The T-DNA contains two cassettes located between the 25 bp Left Border (LB) and 25 bp Right Border (RB) (Figure 2). The two cassettes contain inverted repeats of gene fragments or promoter sequences of four genes. Expression of the inverted repeats triggers a natural degradation of the ribonucleic acid (RNA) transcripts of four endogenous potato genes and thus reduces expression of these genes. This process, referred to as RNA interference (RNAi), is described below.



Figure 2:Design of pSIM1278 T-DNA region.

The transcription of an inverted repeat leads to the production of double-stranded RNA (dsRNA) in the form of a structure known as a hairpin. The dsRNA hairpin is cleaved into small dsRNAs, approximately 21- 24 nucleotides long, via an endogenously occurring protein known as Dicer (Hammond 2005). These mature small dsRNA duplexes contain an interfering antisense strand (the guide strand), which is complementary to the targeted endogenous messenger RNA (mRNA) sequence. The guide strand is incorporated into a multiprotein complex known as the RNA-induced silencing complex (RISC) leading to the targeted destruction of the mRNA transcribed from the four endogenous genes: *Asn1, Ppo5, PhL* and *R1.*

RNA interference (RNAi) is a naturally-occurring RNA-based mechanism that is used by eukaryotes, including plants, to modulate endogenous gene expression as well as destroy foreign RNA, including viral RNA (Parrott et al. 2010). In plants RNAi plays a fundamental role in all aspects of growth and development (Bonnet et al. 2006).

Table 1: Description of the genetic elements contained in the T-DNA of pSIM1278

| **Genetic element** | **Relative bp location on plasmid** | **Size (bp)** | **Source** | **Description, Function & Reference** |
| --- | --- | --- | --- | --- |
| Left border (LB) | 1 - 25 | 25 | Synthetic1 | Site for secondary cleavage to release single-stranded DNA insert from pSIM1278(van Haaren et al. 1989)  |
| Left border region | 26 - 187 | 162 | *S. tuberosum* (var. Ranger Russet) | Supports secondary cleavage at LB |
| Cassette 1 |
| Intervening sequence | 188 - 193 | 6 | *S. tuberosum* | Sequence used for DNA cloning |
| Promoter for the *Agp* gene (*pAgp*)1st copy | 194 -2,453 | 2260 | *S. tuberosum (var. Ranger Russet)* | One of the two convergent promoters driving expression of the inverted repeat fragments of *Asn1* and *Ppo5* |
| Fragment of *Asn1* gene1st copy in antisense orientation | 2,454 - 2,858 | 405 | *S. tuberosum (var. Ranger Russet)* | Forms double stranded RNA with 2nd copy in sense orientation that triggers degradation of *Asn1* RNA to reduce asparagine expression (Chawla et al. 2012)2 |
| 3’-untranslated sequence of *Ppo5* gene1st copy in antisense orientation | 2,859 - 3,002 | 144 | *S. verrucosum* | Forms double stranded RNA with 2nd copy in sense orientation that triggers degradation of *Ppo5* RNA to reduce blackspot development |
| Intervening sequence | 3,003 – 3,008 | 6 | *S. tuberosum* | Sequence used for DNA cloning |
| Spacer-1 | 3.009 – 3,165 | 157 | *S. tuberosum (var. Ranger Russet)* | Sequence between the 1st inverted repeats |
| 3’-untranslated sequence of *Ppo5* gene2nd copy in sense orientation | 3,166 – 3,309 | 144 | *S. verrucosum* | Forms double stranded RNA with 1st copy in antisense orientation that triggers degradation of *Ppo5* RNA to reduce blackspot development |
| Fragment of *Asn1* gene2nd copy in sense orientation | 3,310 – 3,715 | 406 | *S. tuberosum (var. Ranger Russet)* | Forms double stranded RNA with 1st copy in antisense orientation that triggers degradation of *Asn1* RNA to reduce asparagine expression (Chawla et al. 2012)2 |
| Intervening sequence | 3,716 – 3,721 | 6 | *S. tuberosum* | Sequence used for DNA cloning |
| Promoter for the *Gbss* gene (*pGbss*)1st copy | 3,722 – 4,407 | 686 | *S. tuberosum (var. Ranger Russet)* | One of the two convergent promoters driving expression of the inverted repeat fragments of *Asn1* and *Ppo5* |
| Intervening sequence | 4,408 – 4,423 | 16 | *S. tuberosum* | Sequence used for DNA cloning |
| Cassette 2 |
| Promoter for the *pAgo* gene 2nd copy | 4,424 – 6,683 | 2260 | *S. tuberosum (var. Ranger Russet)* | One of the two convergent promoters driving expression of the inverted repeat fragments of *PhL* and *R1* |
| Fragment of *PhL* gene promoter (*pPhL*)1st copy in antisense orientation | 6,684 – 7,192 | 509 | *S. tuberosum (var. Ranger Russet)* | Forms double stranded RNA with 2nd copy in sense orientation that triggers degradation of *PhL* RNA to reduce levels of reducing sugars |
| Fragment of *R1* gene promoter (*pR1*)1st copy in antisense orientation | 7,193 – 7,724 | 532 | *S. tuberosum (var. Ranger Russet)* | Forms double stranded RNA with 2nd copy in sense orientation that triggers degradation of *R1* RNA to reduce levels of reducing sugars |
| Intervening sequence | 7,725 – 7,730 | 6 | *S. tuberosum* | Sequence used for DNA cloning |
| Spacer-2 | 7,731 – 7,988 | 258 | *S. tuberosum (var. Ranger Russet)* | Sequence between the 2nd inverted repeats |
| Fragment of *R1* gene promoter (*pR1*)2nd copy in sense orientation | 7,989 – 8,520 | 532 | *S. tuberosum (var. Ranger Russet)* | Forms double stranded RNA with 1st copy in antisense orientation that triggers degradation of *R1* RNA to reduce levels of reducing sugars |
| Fragment of *PhL* gene promoter (*pPhL*)2nd copy in sense orientation | 8,521 – 9,029 | 509 | *S. tuberosum (var. Ranger Russet)* | Forms double stranded RNA with 1st copy in antisense orientation that triggers degradation of *PhL* RNA to reduce levels of reducing sugars |
| Promoter for the *Gbss* gene (*pGbss*)2nd copy | 9,030 – 9,953 | 924 | *S. tuberosum (var. Ranger Russet)* | One of the two convergent promoters driving expression of the inverted repeat fragments of *PhL and R1* |
| Intervening sequence | 9,954 – 9,962 | 9 | *S. tuberosum* | Sequence used for DNA cloning |
| Right border region | 9,963 – 10,123 | 161 | *S. tuberosum* (var. Ranger Russet) | Supports secondary cleavage at RB site |
| Right border (RB) | 10,124 -10,148 | 25 | Synthetic1 | Site for secondary cleavage to release single-stranded DNA insert from pSIM1278 (van Haaren et al. 1989) |

1The LB and RB sequences (25 bp each) were synthetically designed to be similar to and function like T-DNA borders from *Agrobacterium tumefaciens.*

2ASN1 is referred to as StAst1 in Chawla et al. (2012)

Table : Description of the genetic elements contained in the backbone of pSIM1278

| **Genetic element** | **Relative bp location on plasmid** | **Size (bp)** | **Source** | **Description, Function & Reference** |
| --- | --- | --- | --- | --- |
| Intervening sequence | 10,149 – 10,154 | 6 | Synthetic | Sequence used for DNA cloning |
| Overdrive | 10,155 – 10,184 | 30 | *Agrobacterium tumefaciens* Ti-plasmid | Enhances cleavage of *A. tumefaciens* Right Border site |
| Intervening sequence | 10,185 – 11,266 | 1,082 | *Pseudomonas fluorescens pVS1* | pVS1 backbone1 |
| pVS1 partitioning protein StaA (pVS1 Sta) | 11,267 – 12,267 | 1,001 | *P. fluorescens pVS1* | pVS1 stability1 |
| Intervening sequence | 12,268 – 12,860 | 593 | *P. fluorescens pVS1* | pVS1 backbone1 |
| pVS1 replicon(pVS1Rep)  | 12,861 – 13,861 | 1001 | *P. fluorescens pVS1* | pVS1 replicon region in *Agrobacterium*1 |
| Intervening sequence | 13,862 – 14,099 | 238 | *P. fluorescens pVS1* | pVS1 backbone1 |
| Intervening sequence | 14,100 – 14,270 | 171 | pBR322 | pCambia 1301 backbone1 |
| pBR322 bom | 14,271 – 14,531 | 261 | pBR322 | pBR322 region for replication in *E. coli*1 |
| Intervening sequence | 14,532 – 14,670 | 139 | pBR322 | pCambia 1301 backbone1 |
| Origin of replication for pBR322 (pBR322 ori) | 14,671 – 14,951 | 281 | pBR322 | Bacterial origin of replication1 |
| Intervening sequence | 14,952 – 15,241 | 290 | pBR322 | pCambia 1301 backbone1 |
| Neomycin phosphotransferase II (*nptII*) gene | 15,242 – 16,036 | 795 | Tn5 transposon | Aminoglycoside phosphotransferase (Simpson et al. 1985) |
| Intervening sequence | 16,037 – 16,231 | 195 | Vector DNA | pCambia vector background |
| Terminator of the ubiquitin-3 gene (*tUbi3*) | 16,232 – 16,586 | 355 | *S. tuberosum* | Terminator for i*pt* gene transcription (Garbarino and Belknap 1994) |
| Intervening sequence | 16,587 – 16,937 | 351 | *A. tumefaciens* Ti-plasmid | Sequence for DNA cloning |
| Isopentenyl transferase (*ipt*) gene | 16,938 – 17,660 | 723 | *A. tumefaciens* *Ti-plasmid* | Condensation of AMP and isopentenyl-pyrophosphate to form isopentenyl-AMP, a cytokinin in the plant. Results in abnormal growth phenotypes in plant (Smigocki and Owens 1988) |
| Intervening sequence | 17,661 – 17,672 | 12 | Synthetic DNA | Sequence used for DNA cloning |
| Polyubiquitin promoter (*Ubi7*) | 17,673 – 19,410 | 1,738 | *S. tuberosum (var. Ranger Russet)* | Promoter to drive expression of the *ipt* backbone marker gene |
| Intervening sequence | 19,411 – 19,660 | 250 | Vector DNA | pZP200 vector backbone1 |

1 <http://www.ncbi.nlm.nih.gov/nuccore/AF234297.1>

### 3.2.1 Cassette 1

The first cassette is designed to reduce expression of the *Asparagine synthetase-1 (Asn1)* and *polyphenol oxidase 5* (*Ppo5)* genes in the tuber of the transformed potato. It contains two identical 405-bp fragments of *Asn*1 derived from *S. tuberosum* and two identical 144-bp fragments of *Ppo5* (Figure 2) derived from *S. verrucosum*. The Applicant has indicated >97% identity between the *Ppo5* fragment of *S. verrucosum* and *S. tuberosum*.

The fragments of *Asn1* and *Ppo5* are arranged as inverted repeats separated by a non-coding 157-bp potato nucleotide spacer element (Figure 2).

The *Asn1* and *Ppo5* fragments are arranged between the two convergent potato promoters; the *Agp* promoter of the ADP glucose pyrophosphorylase gene (*pAgp*) and the *Gbss* promoter of the granule-bound starch synthase gene (*pGbss*). The *pAgp* and *pGbss* promoters are well characterised and known to be highly active in potato tubers (Visser et al. 1991; Nakata et al. 1994). These promoters drive expression of the inverted repeats to generate double-stranded RNA which should interfere with the transcription and expression of the *Asn1* and *Ppo5* genes.

The *Asn1* gene catalyses the conversion of the amino acid aspartate into asparagine. Reduced expression of *Asn1* should reduce levels of free asparagine in the potato tuber, which has the potential to reduce the formation of acrylamide in cooked potatoes (Rommens et al. 2008).

In plants, polyphenol oxidases (PPOs) are known for their role in post-harvest browning of fresh produce (see review by Sullivan 2015). Tuber discoloration due to bruising during handling and processing is typically referred to as blackspot or blackspot bruise. This is a post-harvest physiological disorder resulting from physical impact which causes cell rupture or flesh injury. The discoloration is due to leakage of PPO from the vacuoles of injured cells and the subsequent oxidation of polyphenols which triggers precipitation of black melanin (Corsini et al. 1999; Rommens et al. 2006). Blackspot bruise is a major problem in the fresh market and frozen French fry industry, as the affected potatoes require removal of the discoloured area or are discarded (Corsini et al. 1999).

In potato, PPO comprises a multigene family with at least six genes differing in temporal and tissue-specific expression (Thygesen et al. 1995). One member of the PPO gene family, *Ppo5* (called *POT32* in Thygesen et al.1995) is the primary form found in potato tubers, and is the primary message detected in older tubers. While also expressed in roots, no expression was detected in photosynthetic tissues (Thygesen et al. 1995). Reduced expression of *Ppo5* in the potato tuber should lower the extent of blackspot bruise sensitivity.

### 3.2.2 Cassette 2

The second cassette is designed to reduce expression of the *phosphorylase-L* (*PhL*)and *water dikinase* *R1* (*R1*) genes in the tuber of the transformed potato. It contains two identical 509-bp fragments of the *PhL* promoter region (*pPhL*) from *S. tuberosum* and two identical 532-bp fragments of *R1* promoter region (*pR1*) also from *S. tuberosum*. The fragments of *PhL* and *R1* are arranged as inverted repeats separated by a non-coding 258-bp fragment of the potato polyubiquitin gene (spacer 2). As in the first cassette, the *pPhL* and *pR1* fragments are arranged between and transcribed by the potato *Agp* and *Gbss* promoters (Figure 2).

Dormant potato tubers, when subjected to chilling temperatures, undergo a process known as cold sweetening, which results in an increase in sucrose and the reducing sugars, glucose and fructose. Potato tubers contain two starch phosphorylase genes, type L and H, which are believed to be responsible for the complete breakdown of starch. Inhibition of the *phosphorylase-L* (*PhL*) gene has been shown to reduce the accumulation of sucrose and the reducing sugars glucose and fructose in potato tubers stored a low temperature (Kamrani et al. 2011).

The *R1* gene is a regulator of starch catabolism through phosphorylation of alpha-glucans as part of the starch degradation process (Yano et al. 2005). Thus, reducing expression levels of the R1 gene should reduce starch phosphorylation levels and consequently reduce starch degradation, which in turn slows the conversion of starch to reducing sugars, fructose and glucose.

## 3.3 Propagation of E12

Potato is typically propagated asexually through the planting of potato tubers or pieces of tubers. The initial transformed E12 plant, after transfer to soil, produced tubers considered to be the G0 generation. Plants arising from G0 tubers produce the G1 generation of plants and tubers and so on. The E12 generations used in the various analyses is provided in Table 3.

Table : E12 generations used for various analyses

| **Analysis** | **E12 generation used** | **Control(s) used** | **Reference comparators** |
| --- | --- | --- | --- |
| Molecular characterisation (Sections 3.4.1.1 – 3.4.1.3) | G0 T-DNA integrity, absence of backbone, sequencing of junction  | Russet Burbank  | Russet Burbank spiked with plasmid pSIM1278 DNA  |
| RNA levels of four endogenous genes(Section 3.4.3) | G1 tuber, root, leaf, stem, flower | Russet Burbank  | N/A |
| PPO activity(Section 3.4.4) | G2 tuber | Russet Burbank  | N/A |
| Genetic stability (Section 3.5) | G0 leaf and G3 tuber | Russet Burbank  | N/A |
| Compositional analysis (Section 5) | G0, G1 and G2 tubers | Russet Burbank | Russet Burbank and 8 other non-GM potato varieties  |

## 3.4 Characterisation of the genetic modification in the plant.

A range of analyses were undertaken to characterise the genetic modification in E12. These analyses focussed on the nature of the insertion of the introduced genetic elements and whether any unintended re-arrangements may have occurred as a consequence of the transformation procedure.

### 3.4.1 Southern blot analysis: insert copy number, insert integrity and plasmid backbone

Genomic DNA extracted from young leaves of greenhouse-grown, G0 plants of E12 and the control (non-GM Russet Burbank), was used for Southern blot analysis to determine the number of T-DNA insertions, the integrity of the inserted T-DNA and test for the presence or absence of plasmid vector backbone sequences. The genomic DNA was also used to determine the sequence of the junctions and flanking genomic DNA using PCR followed by Sanger DNA sequencing.

#### 3.4.1.1 Number of integration sites

To determine the number of integration sites, genomic DNA was isolated from leaf material of E12 and control plants. DNA was digested with restriction enzyme Nde1 which does not cut within the T-DNA insert, but does cut frequently in the potato genome. The digested DNA was run on a gel, transferred to a membrane and incubated with seven different labelled probes. The seven probes were overlapping and spanned the T-DNA region (Figure 3).



*Figure 3: Southern blot probe locations in T-DNA of plasmid pSIM1278*

In analysing the results, the following points were relevant:

* Since Nde1 does not cut within the T-DNA, a single high molecular weight band is expected for E12 and not for the control when using any of the probes.
* An intact insert will have the same size when using any of the probes.
* The presence of additional bands in E12 but not the control would indicate the presence of a second insert and because Nde1 cuts frequently in the potato genome, it is unlikely that two inserts would be of the same size.
* As the T-DNA is largely derived from potato, the probes will also detect endogenous bands, the number of bands detected depends on the frequency of the sequence in the genome.
* Any bands in the control that are also present in E12 would indicate cross-hybridisation of the T-DNA-specific probe with an endogenous potato sequence.
* T-DNA-specific hybridisation bands were not expected in the control.

For all Nde1 and probe combinations for E12 and the control, the hybridisation bands obtained were consistent with the presence of a single copy of T-DNA inserted at a single site.

#### 3.4.1.2 Insert integrity and sequence

Genomic DNA obtained from the leaves of E12 was used to characterise the DNA sequence at the junction of the insertion site and approximately 3,700 bp of each flanking region. The DNA sequence was determined using PCR followed by Sanger DNA sequencing. Alignment of the E12 flanking sequence to the Michigan State University Spud Database indicates that the integration site is likely to be on chromosome 12. The alignment also revealed a small duplication of chromosomal DNA (approximately 3.2 kb in length) at the insertion site. The host (Russet Burbank) does not have this duplication, indicating the duplication occurred during transformation and integration of the T-DNA. Insertion at this site and the duplication of DNA on chromosome 12 did not disrupt any known potato genes.

Other than the junction sites, the Applicant was unable to provided sequence data for the T-DNA inserted into E12, i.e. sequence across the insertion site. The Applicant has indicated the difficulty in obtaining this data was due to the insert consisting entirely of potato sequences, including inverted repeat sequences of endogenous DNA. A number of factors can influence PCR amplification efficiency, such as the number of base pairs in the inverted repeat, the size of the associated hairpin loop, the sequence (i.e. GC content), reaction conditions, primers and polymerases (Hirao et al. 1992; Akeju and Park 2006; Nelms and Labosky 2011).

An additional level of complexity occurs because the parental variety is tetraploid and has multiple copies of each of the sequences that are targeted for reduced expression, making it difficult to distinguish the E12 insert sequence from the endogenous sequences within the parent variety.



Figure : Structure, digestion pattern and probe locations of the pSIM1278 T-DNA insert

A schematic of the full structure of the pSIM1278 T-DNA insert along with the binding sites for each of the four probes (AGP, ASN, GBS1 and R1). Each probe binds to two different locations within the insert. Restriction sites are indicated along the top of the T-DNA: RV = EcoRV, Hd = HindIII, R1 = EcoR1, and Sc = Sca1.The restriction products that are recognized by the probes are shown within the grey box where the expected sizes are indicated. The actual size of junction bands is shown in parenthesis (sizes of junction bands are estimates, except the 4.5 kb EcoRV band).

The junction and flanking regions of the inserted T-DNA from pSIM1278 have been sequenced and reveal a loss of 24 bp and 119 bp from the Left and Right Border regions, respectively. Additionally, the sequences provided the location of restriction sites in the flanking regions and thus allows for predictions of fragment sizes (Figure 4) which span the junctions of E12. DNA of E12, Russet Burbank (negative control) and a positive control (Russet Burbank spiked with plasmid pSIM1278 DNA) were digested using restriction enzymes EcoRV, HindII, and a double digest using EcoR1 and Sca1. Because the T-DNA insert comprises gene fragments derived from potato genes, the four probes (AGP, ASN, GBS1 and R1) are expected to hybridise to the inserted T-DNA as well as endogenous potato genes.

Three types of bands can be predicted for the above combinations of probes and restriction digests: (1) bands unique to the internal sequence of the T-DNA (internal bands) which should be present in E12 and the positive control; (2) bands unique to the plasmid pSIM1278 DNA only (plasmid bands); and (3) based on sequencing of the flanking regions, bands which are unique and span the junction site (junction bands). Following enzymatic digestions and hybridization with the four probes, the predicted internal, plasmid and junction bands were detected. The probes also hybridised to endogenous potato DNA, but these bands were not related to the transformation event. These results suggest, with the exception of the noted deletions at the Left and Right Border regions, that the inserted T-DNA is structurally intact compared to the plasmid T-DNA region (i.e. rearrangement of the T-DNA was not detected).

#### 3.4.1.3 Plasmid backbone

As noted in Section 3.1, the presence of the vector plasmid backbone was selected against during the development of the GM potato, and thus highly unlikely to be present in line E12.

Plantlets containing the pSIM1278 backbone DNA and expressing *ipt* were discarded based on abnormal growth due to overproduction of the plant hormone cytokinin.

The presence or absence of sequences from the vector plasmid pSIM1278 was assessed using eight backbone-specific probes which cover the span of the backbone DNA in combination with two restriction enzyme digestions (see Figure 5). Genomic DNA of E12, Russet Burbank (negative control) and a positive control were digested with either EcoR1 or EcoR1/Sca1. The positive control comprised Russet Burbank DNA spiked with one copy/genome equivalent of pSIM1278 plasmid DNA added prior to enzymatic digestion.

*Figure 5:**Structure and probe locations for the pSIM1278 backbone and T-DNA*

(A) Linear map of the backbone for pSIM1278. The eight probes used to detect the backbone are indicated below (BB1-8) with the size of the probe indicated in base pairs (bp). The EcorR1 sites and expected band sizes for the pSIM1278 plasmid control are indicated as coloured boxes. (B) Structure of the pSIM1278 insert in E12 is shown. The BB8 probe detects the spacer 2 region of the insert and produces a 0.8 kb band when digested by Sca1.

DNA digested with EcoR1 were hybridised with probes BB1-6. No hybridisations were detected using probes BB1-4 corresponding to backbone DNA in E12, whereas the expected 12.5 kb band was detected in the positive control for each of these blots. The expected 1.57 kb and 5.6 kb fragments were detected in the positive controls for blots probed with BB5 and BB6, respectively. Endogenous bands were detected in all samples probed with BB3 and BB6; these bands were not related to the transformation.

DNA digested with both EcoR1 and Sca1 were hybridised with probes BB7 and BB8. There were no backbone fragments detected in E12 when hybridised with probes BB7 and BB8. The expected 1.57 kb band was detected in the positive controls of the blots probed with BB7 and BB8. The expected 5.6 kb band was detected in the positive control in the blot probed with BB8. The Applicant has indicated that due to cross reactivity with pSIM1278 T-DNA, the spacer 2 element in the T-DNA should be detected by the BB8 probe. Thus, as expected, a 0.8 kb band was detected by the BB8 probe in E12 and positive control, but not in the negative control. Endogenous bands were detected in all samples probed with BB7 and BB8; these bands were not related to the transformation. Based on the above results, there is no evidence of backbone fragments detected in the genome of E12.

### 3.4.2 Open reading frame (ORF) analysis

The Applicant used proprietary Python script to identity all start-to-stop ORFs in the inserted T-DNA and adjacent flanking regions in all six reading frames.

Given the intractability of sequencing the inserted T-DNA (see Section 3.4.1.2), the ORF analysis was based on the DNA sequences of the junction sites and that of the T-DNA region of plasmid pSIM1278. The rationale for this approach to the ORF analysis is not unreasonable because:

* Southern blot analysis (Section 3.4.1.2) indicates the overall structure of the T-DNA region is intact except for some nucleotides lost from the Left and Right Border (expected losses), thus there is no evidence of rearrangement in the inserted T-DNA
* Point mutations, e.g. during cloning of the plasmid in *E. coli* occur at frequency of about 10-10 to 10-9 per nucleotide (Lee et al. 2012) and are thus unlikely to have occurred.
* The T-DNA was designed to create dsRNA leading to degradation of endogenous RNA, thus no unique (novel) proteins would be produced from the T-DNA region.
* If an ORF for a novel protein were identified from this region, its expression is unlikely as the dsRNA produced from the T-DNA would target and degrade the RNA of the novel protein just as it targets and degrades the RNA of the four endogenous genes.

Identified ORFs were analysed using a bioinformatics strategy to determine similarity with known toxins, allergens or other proteins with known biological activity.

The analysis identified 27 ORFs encoding sequences of 30 or more amino acids; ORFs shorter than 29 amino acids were not evaluated since a minimum 35% identity requires at least a match of 29 amino acids over an 80 amino acid sequence. The 35% identity is a recommended criterion for indicating potential allergenicity (Codex 2009). The allergen analyses considered a full-length search, ≥35% identity over 80 amino acids and an 8-mer exact match.

Using the FASTA algorithm (AllergenOnline database[[2]](#footnote-3)) to search for significant alignments with database sequences, no biologically significant matches to known allergens were identified for the identified ORFs.

Potential toxicity was assessed using the National Centre for Biotechnology Information (NCBI) database[[3]](#footnote-4). A BLAST search of the ORFs did not identify any annotated toxins, but did identify 24 proteins that are homologous to the asparagine synthetase gene from potato. These matches are not surprising as a fragment of the asparagine synthetase gene from potato was used to genetically modify line E12. The matches are not themselves toxins and represent bacterial homologs. None of these matches indicate homology with known toxins.

### 3.4.3 Northern blot analyses for reduced expression of four potato genes

Line E12 was transformed using a construct containing inverted repeats of gene fragments intended to reduce the expression of four endogenous genes: *Asn1, Ppo5, PhL* and *R1.* Expression of the inverted repeats should create dsRNA which, through natural plant processes, should degrade the mRNA of the four endogenous genes and thus reduce their expression levels (see Section 3.2). The introduced gene fragments were driven by promoters primarily active in the potato tuber.

RNA extracted from the tuber, leaf, stem, root and flower tissues of field-grown E12 and Russet Burbank (control) plants (G1) was evaluated by northern blot analysis to examine the effectiveness of the suppression of the four endogenous genes.

Results of the northern blot revealed reduced levels of *Asn1* and *Ppo5* RNA in the E12 tubers and a partial reduction of *Asn1* RNA in E12 flowers compared to the tubers and flowers of the control. No differences were observed for *Asn1* and *Ppo5* in any other tissues. There was no observed reduction of *PhL* and *R1* RNA in any of the tissues tested.

### 3.4.4 PPO activity in tubers of line E12

Tubers from field-grown E12 and Russet Burbank (control) plants were assessed for polyphenol oxidase (PPO) activity using a colorimetric assay. Pieces from ten tubers each for E12 and the control were assayed in triplicate by monitoring the conversion of the exogenous dihydroxybeneze substrate, catechol, into melanin which provides a visible colour change. PPO catalyses the oxidation of hydroxyphenolic compounds such as catechol to form o-quinones, leading to the formation of melanin (Muneta 1977; Behbahani et al. 1993; Albarran et al. 2010). Colour change was scored at time zero and again at 15 minutes using the PPO Activity Colorimetric Scoring Scale. After 15 minutes, PPO activity was significantly lower in E12 tubers compared with control tubers. This result is not unexpected, as the intention of the genetic transformation was to lower PPO activity and northern blot analysis (above) revealed reduced levels of *Ppo5* RNA in the tubers of line E12.

## 3.5 Stability of the genetic changes in E12

Potato varieties are maintained from generation to generation through clonal propagation, which means the progeny do not undergo meiotic recombination and would be genetically and phenotypically stable. The Applicant provided Southern blot analysis indicating consistent banding patterns in the first and fourth generations of clonally propagated, E12 plants. For this study, DNA was digested with a single enzyme (EcoRV) which cuts within the inserted T-DNA region and in the adjacent flanking regions. Two separate probes detected predicted fragments which spanned the T-DNA insert and into the flanking region. This study demonstrates stable maintenance of the E12 insert across three cycles of clonal propagation.

## 3.6 Antibiotic resistance marker genes

No antibiotic marker genes are present in E12. The insert sequence analysis (Section 3.4.1.3) showed no plasmid backbone sequences had been integrated into the E12 genome during transformation, i.e. the *nptII* gene, which was used as a bacterial selectable marker gene, is not present in E12.

## 3.7 Conclusion

E12 was generated through *Agrobacterium*-mediated transformation with a single T-DNA containing two expression cassettes. The cassettes contain gene fragments that when transcribed were expected to lead to the suppression of four endogenous potato genes*: Asn1*, *PhL*, *R1*, and *Ppo5*. Comprehensive molecular analyses of E12 indicate there is a single insertion site containing a single complete copy of the T-DNA insert. The introduced genetic elements are stably maintained from one generation to the next. There are no antibiotic resistance marker genes present in the line and no plasmid backbone sequences have been incorporated into the transgenic locus.

Northern blot analyses were used to compare the mRNA levels associated with the four endogenous potato genes particularly in tubers of E12. These analyses demonstrated a reduction in the expression levels of *ASn1* and *Ppo5* but not *PhL* and *R1.*

# 4 Characterisation and safety assessment of new substances

Translation of the dsRNAs to which the introduced sequences (i.e. *Asn, PhL, R1* and *Ppo5*) in E12 give rise is considered unlikely because the hairpin secondary structure that is formed in each case prevents engagement of the 40S ribosomal subunit necessary to initiate translation at the 5’ end of the RNA, and/or it prevents unwinding of the duplex such that the 40S subunit is unable to advance along it (Kozak 1989). Additionally, the dsRNAs are cleaved into small dsRNAs which have limited potential for translation. Therefore, no novel proteins are produced as a consequence of the genetic modification.

FSANZ has thoroughly researched the scientific literature on RNAi in the context of food crops (FSANZ 2013). It is a process that is found naturally in most eukaryotic organisms, including humans. With regard to the RNAi mediators (dsRNAs and small dsRNAs) themselves, the weight of evidence in the published literature on gene silencing does not support the view that these mediators, ingested as part of the human diet, have an impact on human gene expression or are likely to have adverse consequences for humans. Small RNAs in general are abundantly present in the human diet from both plant and animal sources (Ivashuta et al. 2009; Carthew and Sontheimer 2009). There is also no scientific basis for suggesting that, when present as a result of the genetic modification of a plant, they possess different properties or pose a greater risk than those already naturally abundant in foods from conventional plants, animals and microorganisms such as yeasts.

There are no concerns regarding the safety of the dsRNAs and small dsRNAs in E12. A history of safe human consumption of RNAi mediators exists. Potato tubers from E12 are considered to be as safe for human consumption as tubers derived from conventional potato varieties.

# 5 Compositional analyses

The main purpose of compositional analyses is to determine if, as a result of the genetic modification, any unexpected changes have occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analyses can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

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

## 5.1 Key components

There are a number of components in potato that are considered important for compositional analysis. Depending on variety, climate, soil type and farming practice, the composition of potato tubers may vary widely. As a minimum, the key nutrients of potato tubers appropriate for a comparative study include the dry matter, starch, protein, fat, dietary and crude fibre, minerals (crude ash), sugars, and vitamin C. In addition, levels of key toxins such as glycoalkaloids are an important consideration for the assessment of new potato varieties (OECD 2015).

Potato, like tomato and other members of the Solanaceae family, contains several types of toxic alkaloids called glycoalkaloids, which are very bitter in taste. The presence of glycoalkaloids in *Solanum* species is generally believed to be a natural plant defence mechanism against pests and diseases (Friedman 2006). Glycoalkaloids are produced in all parts of the potato plant. Biosynthesis begins in germinating shoots, with leaves attaining maximum glycoalkaloid concentration first, followed by higher concentrations in unripe fruits and flowers (Friedman 2006). Post-harvest exposure of tubers to light, heat and mechanical damage can stimulate glycoalkaloid production (Friedman 2006). The variation in glycoalkaloid content of tubers can be attributed to both genetic effects and the environmental conditions under which the plants are grown and stored following harvest (Friedman 2006; OECD 2015). The widely accepted safety limit for total glycoalkaloids in tubers is 20 mg/100 gm fresh weight (Smith et al. 1996; Health Canada 2010).

Potato also has several anti-nutrients such as protease inhibitors that inhibit the activity of trypsin, chymotrypsin and other proteases. The protease inhibitors are largely inactivated by cooking and thus are mainly a concern if raw or inadequately cooked potatoes are consumed. Lectins are found in virtually all living organisms and some lectins, such as those found in beans, can cause serious health effects in humans and animals. Lectins are inactivated during heating, thus it is the consumption of raw or inadequately cooked potatoes that may cause concern (OECD 2015).

## 5.2 Study design and conduct for key components

**Study submitted:**

2015, Compositional assessment of E12 compared to Russet Burbank**.** Report 15-60-SPS-COMP-01. Simplot Plant Sciences, Boise, Idaho. (unpublished).

Line E12 (G0, G1 or G2) was used for compositional analysis. The control comparator for this study was the parental potato variety, Russet Burbank. The following non-GM potato varieties (reference varieties), with a history of safe use for food and feed, were grown to provide a range of values common to conventional potatoes: Atlantic, Chieftain, IdaRose, Red Norland, Ranger Russet, Snowden, and two proprietary varieties, G and H. The reference varieties and the control variety (Russet Burbank) were used to calculate a tolerance interval to contain, with 95% confidence, 99% of the values contained in the population.

E12 and Russet Burbank were grown at eleven locations in potato growing regions of the United States during the 2009, 2010 and 2011 growing seasons. At least one reference variety was grown at each location. Data on the reference varieties was also collected from 12 other locations during the 2009 – 2011 growing seasons. Trial site design was a randomised complete block comprising three replications of each variety within three blocks at each site. The agronomic practices and pest control measures used were location-specific and were typical for all aspects of potato cultivation including soil preparation, fertiliser application, irrigation and pesticide-based control methods.

Test, control and reference tubers for the compositional analyses were collected from the 2009, 2010 and 2011 trial sites. Samples consisted of five randomly selected tubers from each site and replicate. Selected tubers were about 15 cm in length. Some of the analytical testing was conducted by the J.R. Simplot Co., Boise, Idaho, with the remainder conducted by Covance Laboratories, Inc., Madison, Wisconsin. Methods of composition analysis were based on internationally recognised procedures such as those of the Association of Official Analytical Chemists and USDA, other published methods and those provided by the Applicant. The data from both laboratories was combined in the statistical analysis.

## 5.3 Analyses of key components in tubers

The analytes measured from the raw tubers are listed in Table 4. In addition to the analytes listed below, the Applicant has provided data and analysis on acrylamide levels in cooked E12 tubers. Acrylamide is not a component of potato tubers *per se*, but is a potential by-product of cooking the tubers at high temperatures. The potential for acrylamide production is discussed in Section 5.5.

In total, 38 analyte levels were measured and analysed for the raw tuber. Statistical analyses were performed using JMP statistical discovery software (SAS Institute, Cary, NC). For each analyte, ‘descriptive statistics’ (mean and standard error) were generated. A linear mixed model Analysis of Variance was used by combining data from multiple test years and locations. These results are summarised in Tables 5-11. In assessing the significance of any difference between the mean analyte value for E12 and the control, a P-value of 0.05 was used. This means that approximately 5% of statistically significant differences are expected to occur due to chance alone.

Any statistically significant differences between E12 and the parental variety (Russet Burbank) have been compared to the results of the reference lines and to a combined literature range for each analyte, compiled from published literature for all edible and commercial varieties or parental controls. It is noted that information in the published literature is limited and is unlikely to provide a broad reflection of the natural diversity that occurs within potato. Therefore, even if means fall outside the published range, this is not necessarily a concern.

Table : Tuber composition analytes measured

|  |
| --- |
| Proximates and Fibre (7) |
| Protein | Fat | Ash |
| Crude Fibre | Carbohydrates | Calories |
| Moisture |  |  |
| Vitamins (3) |
| Vitamin B3 | Vitamin B6 | Vitamin C |
| Minerals (3) |
| Copper | Magnesium | Potassium |
| Total Amino Acids (18) |
| Alanine | Histidine | Proline |
| Arginine | Isoleucine | Serine |
| Aspartic acid + Asparagine | Leucine | Threonine |
| Cystine (including Cysteine) | Lysine | Tryptophan |
| Glutamic Acid + Glutamine | Methionine | Tyrosine |
| Glycine | Phenylalanine | Valine |
| Free Amino Acids (4) |
| Asparagine | Aspartic Acid | Glutamine |
| Glutamic Acid |  |  |
| Sugars (2) |
| Fructose + Glucose1 | Sucrose1 |  |
| Toxicants (1) |
| Glycoalkaloids |  |  |

1Analysed in fresh tubers and in tubers stored for various monthly intervals.

### 5.3.1 Proximates and fibre

Results of the proximate and fibre analysis are shown in Table 5. For all the proximate and fibre analytes measured, there was no significant difference between the means for E12 and the means for the control. All means were within both the tolerance interval and combined literature range.

Table 5: Mean value for proximates and fibre in tubers of E12 and control

| **Analyte1** | **Line E12** | **Control** | **P-value2** | **Tolerance Interval** | **Combined literaturerange3**  |
| --- | --- | --- | --- | --- | --- |
| Protein (%) | 2.30 | 2.32 | 0.8796 | 1.26 – 3.59 | 0.7 – 4.6  |
| Fat (%) | 0.10 | 0.10 | 0.9772 | 0 – 0.34 | 0.02 – 0.2 |
| Ash (%) | 1.05 | 1.08 | 0.5871 | 0.39 – 1.89 | 0.44 – 1.9 |
| Crude Fibre (%) | 0.47 | 0.47 | 0.8625 | 0.14 – 0.69 | 0.17 – 3.5 |
| Carbohydrates (%) | 18.5 | 18.5 | 0.9866 | 12.3 – 25.9 | 13.3 – 30.5 |
| Calories (kcal/100 g) | 83.9 | 84.0 | 0.9537 | 59.1 – 115 | 70 – 110.2 |
| Moisture (%) | 78.1 | 78.0 | 0.8815 | 70.3 – 83.9 | 63.2 – 86.9 |

1 Analyte means determined on a fresh weight basis.

2 P-values indicating significant differences are bolded and underlined.

3 Combined literature ranges are from (Talburt et al. 1987; Lisinska and Leszczynski 1989; Horton and Anderson 1992; Rogan et al. 2000).

### 5.3.2 Vitamins and minerals

Results of the vitamin and mineral analysis are shown in Table 6. For all the vitamin and minerals measured, there was no significant difference between the means for E12 and the means for the control. All means were within both the tolerance interval and literature range.

Table 6: Mean value for vitamins and minerals in tubers of E12 and control

| **Analyte1** | **Line E12** | **Control** | **P-value** | **Tolerance Interval** | **Combined literaturerange2** |
| --- | --- | --- | --- | --- | --- |
| Vitamin B3 (mg/100 g) | 1.90 | 1.87 | 0.7264 | 0.92 – 3.12 | 0.09 – 3.1  |
| Vitamin B6 (mg/100 g) | 0.14 | 0.14 | 0.9380 | 0.059 – 0.192 | 0.13 – 0.41 |
| Vitamin C (mg/100 g) | 18.3 | 16.8 | 0.3510 | 0 – 129 | 1 – 54 |
| Copper (ppm) | 1 | 0.96 | 0.6422 | 0.111 – 2.24 | 0.15 – 7 |
| Magnesium (ppm) | 246 | 242 | 0.7238 | 102 – 372 | 112.5 – 550 |
| Potassium (ppm) | 4,777 | 4,681 | 0.6940 | 2,711 – 6,882 | 3,500 – 6,250 |

1 Analyte means determined on a fresh weight basis.

2 Combined literature ranges are from (Talburt et al. 1987; Lisinska and Leszczynski 1989; Horton and Anderson 1992; Rogan et al. 2000).

### 5.3.3 Total amino acids

Analyses of the total amino acids (Table 7) revealed a significant difference in the mean value for asparagine + aspartic acid (lower in E12) and glutamic acid + glutamine (higher in E12) between E12 and the control. These differences were expected because a) the intentional reduction in expression levels of the *Asn1* gene should lower the free asparagine levels and b) asparagine synthetase also functions to deaminate glutamine to glutamate, and therefore reduced levels of asparagine synthetase should increase the level of free glutamine.

In the case of glutamic acid + glutamine, the mean for E12 was within both the tolerance interval and the combined literature range and thus the difference in means is not regarded as biologically meaningful. For asparagine + aspartic acid, the mean for E12 was slightly below the combined literature range but was within the tolerance interval. Again, this indicates that the difference is not biologically significant, especially when considered in light of the fact asparagine and aspartic acid are not essential amino acids and can be synthesised by humans.

The analysis also revealed small but significant increases in the amino acids arginine, cystine and methionine for E12 compared to the control. In the cases of arginine and methionine the relatively small but significantly higher means for E12 are not considered to be biologically meaningful as the means fell within the tolerance and combined literature range. In the case of cysteine, the means for E12 and the control are both lower than the combined literature range but both are within the tolerance interval and hence are also not biologically meaningful.

Table : Mean value (ppm) of total amino acids in tubers of E12 and control

| **Analyte1** | **Line E12** | **Control** | **P-value2** | **Tolerance Interval** | **Combined literaturerange3**  |
| --- | --- | --- | --- | --- | --- |
| Alanine | 718 | 704 | 0.8299 | 100 – 1,335 | 392 – 952  |
| Arginine | 1,080 | 945 | **0.0314** | 368 – 1,922 | 700 – 1,383 |
| Asparagine + Aspartic acid | 2,846 | 5,391 | **<0.0001** | 100 – 13,340 | 3,358 – 7,380 |
| Cystine4(includes cysteine) | 219 | 186 | **0.0455** | 0 – 471 | 286 – 12,500 |
| Glutamic acid + Glutamine | 4,843 | 3,051 | **<0.0001** | 586 – 7,011 | 2,915 – 6,035 |
| Glycine | 1,329 | 1,459 | 0.4886 | 100 – 2,865 | 500 – 1,990  |
| Histidine | 426 | 373 | 0.2571 | 100 – 761 | 133 – 469 |
| Isoleucine | 705 | 687 | 0.7803 | 145 – 1,346 | 525 – 953  |
| Leucine | 1,266 | 1,228 | 0.6831 | 477 – 2,174 | 685 – 1,383 |
| Lysine | 1,111 | 894 | 0.2049 | 100 – 3,363 | 687 – 1,368 |
| Methionine | 418 | 386 | **0.0410** | 100 – 593 | 300 – 500 |
| Phenylalanine | 799 | 787 | 0.7459 | 397 – 1,395 | 552 – 1,087 |
| Proline | 768 | 718 | 0.4801 | 100 – 1,492 | 355 – 1,464 |
| Serine | 575 | 548 | 0.5840 | 100 – 1,362 | 500 – 1,022 |
| Threonine | 659 | 646 | 0.7744 | 226 – 1,315 | 500 – 1,022 |
| Tryptophan4 | 204 | 200 | 0.5724 | 115 – 435  | 436 – 855 |
| Tyrosine | 620 | 562 | 0.3040 | 100 – 1,523 | 457 – 942  |
| Valine | 1,076 | 1,176 | 0.2361 | 100 – 1,860 | 752 – 1,450 |

1 Analyte means determined on a fresh weight basis.

2 P-values indicating significant differences are bolded and underlined.

3 Combined literature ranges are from (Talley et al. 1984; Lisinska and Leszczynski 1989; Rogan et al. 2000; OECD 2002).

4 Cystine and tryptophan measured in 2011 only.

### 5.3.4 Free amino acids

Analysis of the free amino acids (Table 8) revealed a significant difference in the mean levels of asparagine (decreased) and glutamine (increased) in the tubers of E12 compared to Russet Burbank, while the mean levels of aspartic acid and glutamic acid were unchanged. However, the mean concentration of free asparagine and glutamine were within the tolerance interval and the combined literature range and thus were considered equivalent to conventional potatoes.

Table 8: Mean value (ppm) of free amino acids in tubers of E12 and control

| **Analyte** | **Line E12** | **Control** | **P-value2** | **Tolerance Interval** | **Combined literaturerange3**  |
| --- | --- | --- | --- | --- | --- |
| Free Asparagine | 618 | 2,421 | **<0.0001** | 500 – 4,952  | 312 – 6,890 |
| Free Aspartic acid | 448 | 439 | 0.9073 | 100 – 1,411 | 64 – 752 |
| Free Glutamine | 1997 | 1111 | **<0.0001** | 100 – 3,449 | 440 – 5,3964 |
| Free Glutamic acid | 460 | 466 | 0.9008 | 100 – 1,192 | 450 – 742 |

1Analyte means determined on a fresh weight basis.

2P-values indicating significant differences are bolded and underlined.

3Combined literature ranges are from (Davies 1977; Lisinska and Leszczynski 1989; Shepherd et al. 2010).

4 For glutamine, the maximum value from the CLR high level is reported as 5,396 ppm from the mean of four sites (Davies 1977). A value of 18,244 ppm from a single site was not included because it appeared to be an outlier.

### 5.3.5 Reducing sugars and sucrose content

Following harvest of commercial potato tubers at the beginning of autumn, while some tubers may immediately enter the food supply, others will be held for a period in cold storage to allow for year-round release as market demand requires. The temperature of storage and the processes of both pre- and post-storage treatments are critical to ensuring minimal production of reducing sugars that lead to spoilage.

The intent of the genetic modification was to suppress the *PhL* and *R1* genes and therefore reduce the accumulation of sucrose and the reducing sugars glucose and fructose in potato tubers (see Section 3.2.2). However, as shown in the analysis of transcripts of these genes (Section 3.4.3) there was no suppression when compared to the Russet Burbank parent control. It would therefore be expected that the level of reducing sugars and sucrose would not be reduced.

While there was a general tendency for E12 tubers to show lower levels of reducing sugars and sucrose measured when fresh and after one, three and five months storage compared to control tubers (Table 9), the mean level of the reducing sugars was only statistically lower for E12 at the one month storage time-point. This indicates a lack of any trend in the results and suggests that the results at one month storage are outliers.

Table : Mean value (%) of reducing sugars and sucrose in tubers of E12 and control at harvest and after storage

| **Time point** | **Line E12** | **Control** | **P-value1** | **Tolerance Interval2** | **Combined literaturerange3**  |
| --- | --- | --- | --- | --- | --- |
| Fructose & Glucose (reducing sugars)4 |
| Fresh5 | 0.16 | 0.195 | 0.0760 | 0 – 0.307  | 0.018 – 0.803 |
| 1 Month storage6 | 0.107 | 0.162 | **0.0051** | 0 – 0.307  | 0.018 – 0.803 |
| 3 Months storage7 | 0.249 | 0.290 | 0.2001 | 0 – 0.307  | 0.018 – 0.803 |
| 5 Months storage8 | 0.145 | 0.190 | 0.2885 | 0 – 0.307  | 0.018 – 0.803 |
| Sucrose |
| Fresh5 | 0.213 | 0.179 | 0.2115 | 0 – 0.315  | 0.0397 – 1.39 |
| 1 Month storage6 | 0.132 | 0.168 | **0.0123** | 0 – 0.315  | 0.0397 – 1.39 |
| 3 Months storage7 | 0.0734 | 0.0793 | 0.5109 | 0 – 0.315  | 0.0397 – 1.39 |
| 5 Months storage8 | 0.0882 | 0.0792 | 0.5554 | 0 – 0.315  | 0.0397 – 1.39 |

1P-values indicating significant differences are bolded and underlined.

2Tolerance intervals provided were calculated across all time points.

3Combined literature ranges provided are from (Amrein et al. 2003; Vivanti et al. 2006) and include all time points.

4Analyte means determined on a fresh weight basis.

5Tubers from fresh time point were analysed from all sites in 2011.

6Tubers from 1-month time point were analysed from all sites in 2009 and 2010.

6Tubers from 3-month time point were analysed from all sites in 2009 and 2010 and only from two sites in 2011.

8Tubers from 5-month time point were analysed from all sites in 2009 and 2010.

### 5.3.6 Glycoalkaloids in tubers

The mean concentration of total glycoalkaloids in tubers from E12 was not significantly different from the control, and fell within the tolerance interval and the combined literature range (Table 10). Glycoalkaloids are toxins commonly found in Solanaceous crops, including potato, and 95% of the total glycoalkaloids in potato tubers consists of α-solanine and α-chaconine (OECD 2015). The widely accepted safety limit for total glycoalkaloids in tubers is 20 mg/100 gm fresh weight (Smith et al. 1996; Health Canada 2010), and both E12 and the control were well below this limit.

Table : Mean value (mg/100 g) of glycoalkaloids in tubers of E12 and control

| **Analyte1** | **Line E12** | **Control** | **P-value2** | **Tolerance Interval** | **Combined literaturerange**  |
| --- | --- | --- | --- | --- | --- |
| Glycoalkaloids | 5.68 | 7.40 | 0.0524 | 0 – 33.1  | Not available |

1Analyte mean determined on a fresh weight basis.

2P-values indicating significant differences are bolded and underlined.

### 5.3.7 Summary of analysis of key components

A summary of the statistically significant differences in the analyte levels found between tubers of E12 and the control is provided in Table 11.

Table : Summary of analyte levels found in tubers of E12 that are significantly different (P < 0.05) from the control

| **Analyte** | **E12** **mean** | **Control mean** | **Difference between E12 & control means** | **Diff between max and min in control** | **E12 within tolerance interval?** | **E12 within literature range?** |
| --- | --- | --- | --- | --- | --- | --- |
| Arginine (ppm) | 1,080 | 945 | 135 | 1223 | yes | yes |
| Asparagine + Aspartic acid (ppm) | 2,846 | 5,391 | 2545 | 6138 | yes | no (lower) |
| Cystine (ppm)(includes cysteine) | 219 | 186 | 33 | 133 | yes | no (lower1) |
| Glutamic acid + Glutamine (ppm) | 4,843 | 3,051 | 1792 | 2142 | yes | yes |
| Methionine (ppm) | 418 | 368 | 50 | 431 | yes | yes |
| Free Asparagine (ppm) | 618 | 2,421 | 1803 | 3208 | yes | yes |
| Free Glutamine (ppm) | 1997 | 1111 | 886 | 1095 | yes | yes |

1The mean level of cystine for both E12 and the control were below the range recorded in the literature, cystine was measured in 2011 only.

## 5.4 Conclusions of the compositional analyses

Detailed compositional analyses were done to establish the nutritional adequacy of tubers from E12 and to characterise any unintended compositional changes. Analyses were done of proximates, fibre, vitamins, minerals, total amino acids, free amino acids, sucrose, reducing sugars (fructose and glucose), and glycoalkaloids. The levels were compared to levels in: a) the non-GM potato parental line, Russet Burbank; b) a reference range compiled from results taken for Russet Burbank and eight other non-GM commercial varieties grown under the same or similar conditions; and c) levels recorded in the literature. Only seven (summarised in Table 11 and excluding the reducing sugars and sucrose that were recorded over a time period and gave inconsistent results) of the 38 analytes considered deviated from the control in a statistically significant manner; two of these differences (free asparagine and free glutamine) were expected due to the genetic modification. However, the mean levels of both of these analytes fell within the reference range, and all but two analytes fell within the combined literature range. The two analytes (asparagine + aspartic acid and cysteine) were both lower than the combined literature range and as discussed above, were not considered biologically significant. Additionally, for all analytes listed in Table 11, the difference between the mean of E12 and the control was smaller than the variation within the control. It can therefore be concluded that tubers from E12 are compositionally equivalent to tubers from conventional potato varieties.

## 5.5 Reduced acrylamide potential

The intent of the genetic modification was to reduce the expression of four potato genes with the dual aims of reducing blackspot bruising and the potential to form acrylamide during cooking. The Applicant has provided data showing reduced *Ppo5* RNA (Section 3.4.3) and reduced PPO activity (Section 3.4.4) in the E12 tubers, which should lead to a reduction in blackspot bruising.

E12 tubers showed a reduction of *Asn1* mRNA (Section 3.4.3) and a concomitant and significant reduction in free asparagine levels (Section 5.3.4) compared to the control. There was no observable reduction in *PhL* and *R1* mRNA (Section 3.4.3), and no significantly consistent lowering of reducing sugars (Section 5.3.5).

Acrylamide levels were determined from freshly harvested tubers and tubers which were stored for 2, 3, 5, 6 and 7 months; the tubers were processed into cooked fries. Acrylamide levels in the cooked E12 tubers were reduced by more than 50% at every stage tested compared to the control (Table 12). This indicated that despite the expression of *PhL* and *R1* not being reduced, the reduction in expression of *ASn1* was sufficient by itself to produce the desired trait (reduced acrylamide production) in cooked products (fries) of E12.

Table : Mean acrylamide (ppb) in cooked E12 and control tubers at harvest and after storage

| **Time point** | **Line E12** | **Control** | **Percent Reduction** |
| --- | --- | --- | --- |
| Fresh1 | 162 | 494 | 67.2 |
| 2 Month storage2 | 237 | 779 | 69.6 |
| 3 Months storage3 | 315 | 814 | 61.3 |
| 5 Months storage4 | 82 | 230 | 64.3 |
| 6 Months storage5 | 192 | 401 | 52.1 |
| 7 Months storage6 | 192 | 661 | 71.0 |

1Tubers from the fresh time point were analysed from all sites in 2010 and 2011.

2Tubers from the 2-month time point were analysed from all sites in 2009.

3Tubers from the 3-month time point were analysed from all sites in 2010 and 2011.

4Tubers from the 5-month time point were analysed from all sites in 2009.

5Tubers from the 6-month time point were analysed from all sites in 2010.

6Tubers from the 7-month time point were analysed only from one site in 2009.

# 6 Nutritional impact

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

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

Where a GM food has been shown to be compositionally equivalent to conventional varieties, the evidence to date indicates that feeding studies will add little to the safety assessment and generally are not warranted (see e.g. OECD 2003; Bartholomaeus et al. 2013; Herman and Ekmay 2014). E12 is the result of a genetic modification with the ultimate intention of reducing blackspot bruising and reducing acrylamide levels in potatoes processed at high temperatures. To achieve this outcome, the genetic modification targeted the reduced expression of four endogenous genes; no novel proteins are produced in E12. The extensive compositional analyses of the potato tubers that have been undertaken to demonstrate the nutritional adequacy of line E12 indicate it is equivalent in composition to conventional potato cultivars.

Acrylamide is not a component of potato *per se*, it is a product that can form in potato and other starchy foods during high-temperature cooking processes, such as deep frying. At high temperature cooking, acrylamide can form in the presence of reducing sugars (fructose and glucose) and free asparagine that are naturally present in food.

The Applicant states that reducing acrylamide potential in potatoes is desirable because acrylamide presents a potential health risk for consumers (FDA 2016).

The introduction of food from E12 into the food supply is therefore expected to have little nutritional impact and, as such, no additional studies, including animal feeding studies, are required.

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1. <http://agriculture.vic.gov.au/agriculture/horticulture/vegetables/potatoes/potato-varieties> (accessed April 2016) [↑](#footnote-ref-2)
2. available through the Food Allergy Research and Resource Program (FARRP) via the University of Nebraska (<http://www.allergenonline.org/databasefasta.shtml>) [↑](#footnote-ref-3)
3. <http://www.ncbi.nlm.nih.gov/> [↑](#footnote-ref-4)