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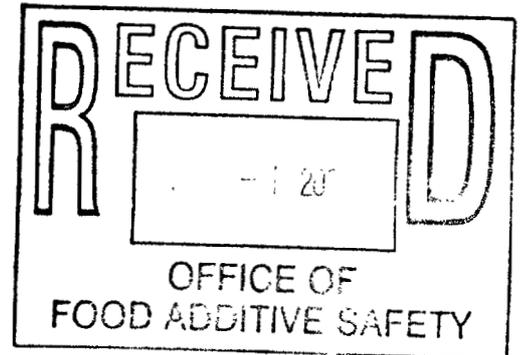


**ORIGINAL SUBMISSION**

000001

May 30, 2006

Robert Martin, Ph.D.  
Deputy Division Director  
Division of Biotech and GRAS Notice Review  
Office of Food Additive Safety (HFS-255)  
Center for Food Safety and Applied Nutrition  
Food And Drug Administration  
5100 Paint Branch Parkway  
College Park, MD 20740-3835



Dear Dr. Martin,

We are hereby submitting, in triplicate, a generally recognized as safe (GRAS) notification, in accordance with proposed 21 C.F.R. § 170.36, for Novozymes' asparaginase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding an asparaginase from *Aspergillus oryzae*. The enzyme preparation is intended for use in the food industry to reduce the formation of acrylamide in the production of fabricated potato chips, and wheat dough based products such as cookies and crackers.

Please contact me by direct telephone at 919 494-3151, direct fax at 919 494-3420 or email at [lobg@novozymes.com](mailto:lobg@novozymes.com) if you have any questions or require additional information.

Sincerely,

Lori Gregg  
Regulatory Specialist

Enclosures (3 binders)

Novozymes North America, Inc  
77 Perry Chapel Church Road  
P.O. Box 576  
Franklinton, North Carolina 27525

000002

May 30, 2006



RE: GRAS Notification - Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) Novozymes North America Inc. hereby claims that asparaginase preparations produced by submerged fermentation of *Aspergillus oryzae* expressing the gene encoding an asparaginase from *A. oryzae* are Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed § 170.36 (c)(1)(i) *The name and address of the notifier.*

Novozymes North America Inc.  
77 Perry Chapel Church Rd., Box 576  
Franklinton, NC 27525

Proposed § 170.36 (c)(1)(ii) *The common or usual name of notified substance.*

Asparaginase preparations produced by *Aspergillus oryzae* expressing the gene encoding an asparaginase from *A. oryzae*.

Proposed § 170.36 (c)(1)(iii) *Applicable conditions of use.*

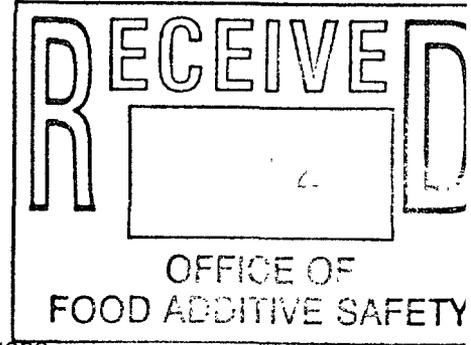
The enzyme preparation is intended for use in the food industry as a processing aid to reduce formation of acrylamide in fabricated potato chips and wheat dough based snack foods such as cookies and crackers. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements for normal production following Good Manufacturing Practices.

Proposed § 170.36 (c)(1)(iv) *Basis for GRAS determination.*

This GRAS determination is based on scientific procedures.

Proposed § 170.36 (c)(1)(v) *Availability of information.*

A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes a safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying upon request.



*May 30, 2006*  
Date

John Carroll  
Director, Regulatory Affairs

Novozymes North America, Inc.  
77 Perry Chapel Church Road  
P.O. Box 576  
Franklinton, North Carolina 27525

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**An asparaginase preparation produced by  
*Aspergillus oryzae* expressing the  
*Aspergillus oryzae* asparaginase gene**

**Lori Gregg, Regulatory Affairs, Novozymes North America, Inc., USA  
Peter Hvass, Regulatory Affairs, Novozymes A/S, Denmark**

May 2006

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## 1. GENERAL INTRODUCTION

The subject of this notification is an asparaginase preparation produced by submerged fermentation of an *Aspergillus oryzae* microorganism carrying the gene coding for asparaginase from *Aspergillus oryzae*. The Novozymes A/S trade name used for the asparaginase preparation is Acrylaway<sup>®</sup>. Asparaginase is intended to be used to convert asparagine to aspartic acid in order to reduce the formation of acrylamide in production of fabricated potato chips, and wheat dough based products such as cookies and crackers.

The active enzyme is an asparaginase (EC 3.5.1.1, CAS 9015-68-3). Asparaginase can be found in the cells of plants, animals, bacteria and fungi (Ref. 1) (See section 7.3).

The information provided in the following sections is the basis for our determination of general recognition of safety of this enzyme preparation produced by *A. oryzae* expressing a gene encoding an asparaginase from *A. oryzae*. Our safety evaluation in Section 7 includes an evaluation of the production strain including an evaluation of safe strain lineage, the donor strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure to the preparation.

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Ref. 2, 3). The production organism for this asparaginase, *A. oryzae*, is discussed in Sections 2 and 7. The production organism is derived from an *A. oryzae* strain that has been used for many years to manufacture enzymes for use in food. Also, Novozymes has used *A. oryzae* strain 1560 for many years to develop improved strains by genetic modification. In section 7.1.1, we show the basis for a safe strain lineage for this production strain following the procedure outlined in Pariza and Johnson 2001 (Appendix 1).

An essential aspect of the safety evaluation of food components derived from genetically modified organisms is the identification and characterization of the inserted genetic material (Ref. 4-9). The genetic modifications used to construct the production microorganism are well defined and are described in Section 2. The recipient strain, *A. oryzae* BECh2, is the same strain that was used in the construction of Novozymes' production strains for a modified lipase, glucose oxidase, and phospholipase which are the subjects of GRN Nos. 103, 106 and 142, respectively. Safety studies including 13 week oral toxicity in rats, Ames test, and human lymphocyte cytogenetic assay, were performed on those preparations. Except for the coding sequence for the enzyme itself, the DNA that was introduced into the recipient strain to construct the asparaginase production strain is essentially the same as the introduced DNA in the GRN 103 and 142 production strains. Novozymes also has unpublished toxicological data (comprising of a 13 week acute oral toxicity in rats, an Ames test, and an In vitro human lymphocyte cytogenetic assay) on a phytase preparation and a xylanase preparation produced by an



*A. oryzae* strain constructed from *A. oryzae* BECh2. All of these studies support the view that strains derived from *A. oryzae* BECh2 can be safely used to manufacture food enzymes. Furthermore, Novozymes has published and unpublished toxicological data on related strains of *A. oryzae* used in the manufacture of food enzymes (see section 7.1.1). All of these studies confirm the safety of *A. oryzae* as a production organism for food enzymes.

## 2. PRODUCTION MICROORGANISM

### 2.1 Production Strain

The *A. oryzae* strain, designated pCaHj621/BECh2#10, was constructed by plasmid transformation of the recipient strain, designated BECh 2 (see Section 2.2). This genetically modified production organism complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms (Ref. 10). It also meets the criteria for a safe production microorganism as described by Pariza and Foster (Ref. 3) and several expert groups (Ref. 2, 4-9).

The asparaginase expression plasmid, pCaHj621, used in the strain construction contains strictly defined fungal chromosomal DNA fragments and synthetic DNA linker sequences. The specific DNA sequences include: a gene encoding an *A. oryzae* asparaginase enzyme; an *Aspergillus nidulans* selectable marker gene, *amdS* (acetamidase) (Ref. 11); well-characterized noncoding regulatory sequences including the *A. niger* terminator (Ref. 12), the *A. niger* neutral amylase II (NA2) promoter (Ref. 12), and *A. nidulans* triose phosphate isomerase gene (Ref. 14); a 1163 bp fragment from the *Escherichia coli* cloning vector pUC19 (Ref. 15), and the *Saccharomyces cerevisiae* URA3 (Ref. 16) promoter (Pura3), coding sequence (URA3) and terminator (Tura3).

### 2.2 Recipient Organism

The recipient microorganism, designated BECh 2, used in the construction of the asparaginase production strain is an amylase negative, alkaline protease (alp) negative, neutral metalloprotease I (Npl) negative, cyclopiazonic acid deficient, kojic acid impaired derivative of the fully characterized, well-known industrial production strain of *A. oryzae* (Ahlburg) Cohn. The strain was obtained from the Institute for Fermentation, Osaka, Japan (IFO) and is designated strain IFO 4177 (synonym A1560). This classification of A1560 as *A. oryzae* has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland (Ref. 17).

The host strain, *A. oryzae* BECh 2, was constructed from A1560 through the following steps:

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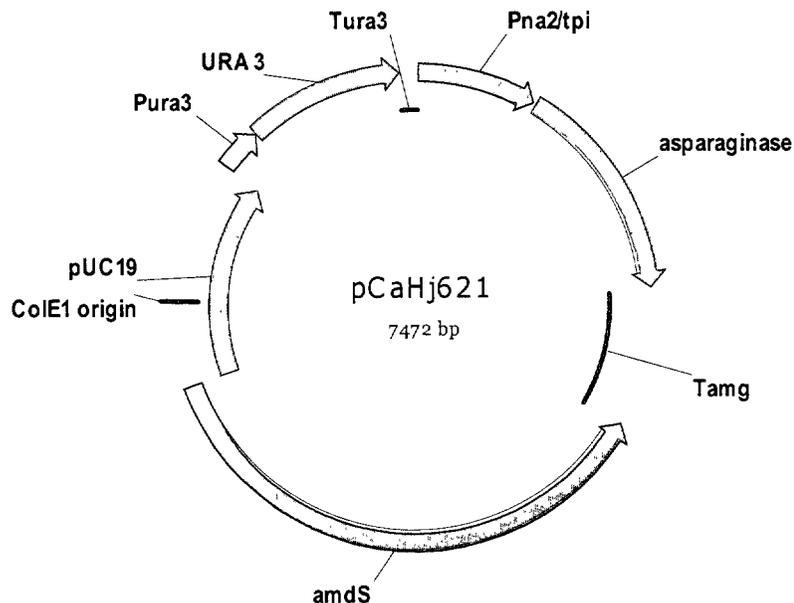
- 1) **HowB101**, a *pyrG* mutant of *A. oryzae* A1560, was obtained by homologous recombination with linearized DNA from the 5' and 3' flanking sequences of the *A. oryzae pyrG* gene.
- 2) **HowB424** was obtained by transformation of HowB101 with linear DNA, containing the entire *pyrG* gene inserted into the *A. oryzae* TAKA-amylase gene, followed by selection for uridine auxotrophy. HowB424 was selected because it showed no secretion of TAKA-amylase.
- 3) **HowB425**, a spontaneous *pyrG* mutant of HowB424, was isolated by plating of spores on agar plates containing 5-fluoro-orotic acid (5-FOA).
- 4) **JaL142** was obtained by transformation of HowB425 with linear DNA, containing the entire *pyrG* gene inserted into the *A. oryzae* alkaline protease gene, and selection for uridine auxotrophs.
- 5) **JaL151**, a spontaneous *pyrG* mutant of JaL142, was isolated by plating of spores on agar plates containing 5-fluoro-orotic acid (5-FOA).
- 6) **JaL228** obtained by transformation of JaL151 with linear DNA, containing the entire *pyrG* gene inserted into the *A. oryzae* neutral metalloprotease I gene, and selection for uridine auxotrophs.
- 7) **BECh 1** obtained by  $\gamma$  irradiation of spores of JaL228 and screening of a mutant deficient in cyclopiazonic acid synthesis.
- 8) **BECh 2** obtained by U.V irradiation of spores of BECh 1 and screening of a mutant deficient in Kojic acid synthesis

Only *A. oryzae* DNA was transformed into the *A. oryzae* A1560 during the development of the host strain BECh2, so only homologous gene manipulations have been applied.

### 2.3 Asparaginase Expression Plasmid

The 7472 bp expression plasmid, pCaHj621, used to transform the *A. oryzae* host strain, BECh 2, contains the gene elements outlined below. This expression plasmid is essentially the same as the expression plasmid, pCaHj559, used in construction of the production strain for the modified lipase, which is the subject of GRN No. 103 and the expression plasmid, pPFJo142, used in the construction of production strain for the phospholipase, which is the subject of GRN No. 142. The only difference is the use of the double promoter in pPFJo142 plasmid for the phospholipase and the coding sequence of the enzyme itself.

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Position (bp)	Size (bp)	Element	Origin
1-616	616	Pna2/tpi	<i>A. niger</i> BO1
617-627	9	Linker	Synthetic
628-1761	1134	Asparaginase	<i>A. oryzae</i>
1762-1778	17	Linker	Synthetic
1779-2476	698	Tamg	<i>A. niger</i> BO1
2477-5201	2725	amdS	<i>A. nidulans</i> .
5202-6364	1163	pUC19	<i>E. coli</i> .
6365-6587	223	Pura3	<i>S. cerevisiae</i> .
6588-7388	801	URA3	<i>S. cerevisiae</i> .
7389-7472	84	Tura3	<i>S. cerevisiae</i> .

Pna2/tpi is the neutral amylase II promoter from *Aspergillus niger*. The 5' nontranslated part of this promoter has been replaced with the 5' nontranslated part of the *Aspergillus nidulans* triose phosphate isomerase (TPI) promoter (position 550-616).

Tamg is the amyloglycosidase terminator of *Aspergillus niger*.

amdS is the acetamide gene (including promoter and terminator) from *A. nidulans*.

pUC19 is a fragment of the pUC19 vector including the origin of replication. The origin of replication initiation site (colE1 origin) is in position 5637.

Pura3, URA3 and Tura 3 are the *S. cerevisiae* URA3 promoter, coding sequence and terminator respectively.

## 2.4 Stability of the Introduced Genetic Sequences

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The presence of the introduced DNA sequences was determined by Southern hybridization to assess the stability and potential for transfer of genetic material as a



component of the safety evaluation of the production microorganism (Ref. 4-9). The transforming DNA is stably integrated into the *A. oryzae* chromosome and, as such, is poorly mobilizable for genetic transfer to other organisms and is mitotically stable (Ref. 10).

### 2.5 Antibiotic Resistance Gene

The introduced DNA does not contain antibiotic resistance genes.

### 2.6 Absence of Production Organism in Product

The absence of the production organism is an established specification for the commercial product. The production organism does not end up in food and therefore the first step in the safety assessment as described by IFBC (Ref. 4) is satisfactorily addressed.

## 3. MANUFACTURING PROCESS

This section describes the manufacturing process for the asparaginase which follows standard industry practices (Ref. 18-20). The quality management system used in the manufacturing process for the asparaginase complies with the requirements of ISO 9001. It is also manufactured in accordance with current good manufacturing practices.

### 3.1 Raw Materials

The raw materials used in the fermentation and recovery process for the asparaginase enzyme concentrate are standard ingredients used in the enzyme industry (Ref. 18-20). The raw materials conform to Food Chemicals Codex specifications except those raw materials which do not appear in the FCC. For those not appearing in the FCC, internal specifications have been made in line with FCC requirements. On arrival at Novozymes A/S, the raw materials are sampled by the Quality Control Department and subjected to the appropriate analyses to ensure their conformance to specifications.

The antifoams used in fermentation and recovery are used in accordance with the Enzyme Technical Association submission to FDA on antifoams and flocculants dated April 10, 1998. The maximum use level of these antifoams in the product is less than 1%.

### 3.2 Fermentation Process

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The asparaginase is manufactured by submerged fed-batch pure culture fermentation of the genetically modified strain of *A. oryzae* described in Section 2. All equipment is carefully designed, constructed, operated, cleaned, and maintained



so as to prevent contamination by foreign microorganisms. During all steps of fermentation, physical and chemical control measures are taken and microbiological analyses are done to ensure absence of foreign microorganisms and confirm strain identity.

### 3.2.1 Production Organism

Each batch of the fermentation process is initiated with a lyophilized stock culture of the production organism, *A. oryzae*, described in section 2. Each new batch of the stock culture is thoroughly controlled for identity, absence of foreign microorganisms, and enzyme-generating ability before use.

### 3.2.2 Criteria for the Rejection of Fermentation Batches

Growth characteristics during fermentation are observed both macroscopically and microscopically. Samples are taken from both the seed fermentor and the main fermentor before inoculation, at regular intervals during cultivation, and before transfer/harvest. These samples are tested for microbiological contamination by microscopy and by plating on a nutrient agar followed by a 24-48 hour incubation period.

The fermentation is declared "contaminated" if one of the following conditions are fulfilled:

1. Infection is observed in 2 or more samples by microscopy
2. Infection is observed in two successive agar plates at a minimum interval of 6 hours

Any contaminated fermentation is rejected.

### 3.3 Recovery Process

The recovery process is a multi-step operation which starts immediately after the fermentation process and consists of both the purification and the formulation processes.

#### 3.3.1 Purification Process

The enzyme is recovered from the culture broth by the following series of operations:

1. Pretreatment - pH adjustment
2. Primary Separation - vacuum drum filtration

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3. Concentration - ultrafiltration and/or evaporation
4. Pre- and Germ Filtration - for removal of residual production strain organisms and as a general precaution against microbial degradation
5. Preservation and Stabilization of the liquid enzyme concentrate
6. Final concentration – evaporation and/or ultrafiltration if enzyme concentration is too low to reach target yield

### 3.3.2 Formulation and Standardization Processes

The stabilized concentrate is blended with water and glycerol and preserved with sodium benzoate and potassium sorbate. The product is standardized according to the product specification.

### 3.4 Quality Control of Finished Product

The final products are analyzed according to the specifications given in section 5.

## 4. ENZYME IDENTITY

Key enzyme and protein chemical characteristics of the phospholipase are given below:

Classification	Asparaginase
IUB nomenclature:	L-Asparagine amidohydrolase
IUB No.:	3.5.1.1
CAS No.:	9015-68-3
Specificity:	hydrolyses the amide of L-Asparagine to form aspartic acid and ammonia
Amino acid sequence:	the total nucleotide and amino acid sequences have been determined

## 5. COMPOSITION AND SPECIFICATIONS

The asparaginase enzyme preparation is presently available in a formula for use in food applications. The Novozymes A/S trade name used for the asparaginase preparation is Acrylaway®.

## 5.1 Quantitative Composition

Acrylaway L has the following typical composition:

Enzyme solids (TOS)	approx.	4 %
Glycerol	approx.	50 %
Water	approx.	46 %
Sodium benzoate	approx.	0.3 %
Potassium sorbate	approx.	0.1 %

Acrylaway L has a typical activity of 3500 ASNU/g. Asparaginase converts L-asparagine into L-aspartate and ammonia. One ASNU is the amount of enzyme that produces 1 micromole ammonia per minute under specific reaction conditions.

## 5.2 Specifications

The asparaginase preparation complies with the purity criteria recommended for enzyme preparations as described in *Food Chemicals Codex* (Ref. 21). In addition, it also conforms to the General Specifications for Enzyme Preparations Used in Food Processing as proposed by the Joint FAO/WHO Expert Committee on Food Additives in Compendium of Food Additive Specifications (Ref. 22).

## 6. APPLICATION

### 6.1 Mode of Action

The enzyme in Acrylaway is an asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1). The enzyme hydrolyzes the amino acid asparagine to aspartic acid by hydrolyzing the amide in asparagine to the corresponding acid (=aspartic acid). Apart from asparagine, asparaginase only acts on glutamine and it has no activity on other amino acids. It has no activity on asparagine residues in peptides or proteins.

Asparaginases are produced by a variety of microbes, including both bacteria and fungi (Ref. 23). In many of these microbes, two asparaginases are found, a cytoplasmic form and a periplasmic or extra-cellular form. In the few cases where they have been compared, the extra-cellular asparaginases have a higher affinity for asparagines (Ref. 24, 25). Several asparaginases from bacteria are well characterized (Ref. 26). The asparaginase in Acrylaway is an extra-cellular enzyme.

Acrylaway is to be used for acrylamide reduction in various food applications. Acrylamide is formed as a reaction product from asparagine and reducing sugars when food products are baked or fried at temperatures above 120°C. Both asparagine and reducing sugars are commonly found in many food raw materials. By using asparaginase, the asparagine content will be reduced and hereby also the acrylamide in the final product. Typical dough based applications include: biscuits,



crackers, crisp bread, tortilla chips, fabricated potato chips, pretzels, bread, etc. The enzyme can also be used for direct treatment of cut potatoes for production of French fries, sliced potato chips, potato flakes and potato granules. (Appendix 2)

## 6.2 Use Levels

The enzyme preparation is used at minimum levels necessary to achieve the required asparagine/acrylamide reduction and according to requirements for normal production following cGMP.

### Dough-based products

For the dough based applications, the recommended dosage of Acrylaway is from 200 up to 2500 ASNU per kg processed food, corresponding to 6-70 g of Acrylaway per 100 kg processed food.

### Cut pieces of vegetables

For the treatment of potatoes (e.g. French fries, sliced potato chips) dosages calculated per kg final product is up to approx. 2000 ASNU.

For French fries, the enzyme treatment will potentially be done as a continuous process where potato strips or slices are dipped in an enzyme bath of a concentration up to 12,000 ASNU/l water for a specified holding time. The enzyme bath will be reused for a certain period of time. Water pick-up by the treated potatoes is assumed to be maximum 5% corresponding to a dosage of 600 ASNU/kg treated potatoes. Calculated per kg parfried product going out of the factory enzyme dosage is 850 ASNU/kg and calculated per kg final product prepared for consumption enzyme dosage is 1420 ASNU/kg. Above calculations are based upon an estimated mass balance of 1 kg treated potato strips giving 0.7 kg parfried product giving 0.42 kg product ready for consumption.

For sliced potato chips production the estimated balance is that 1 kg of treated potato slices will give 0.33 kg of final product. With a treatment in an enzyme bath containing 12,000 ASNU/l and 5% water pick-up this corresponds to 600 ASNU/kg treated potatoes and 1820 ASNU/kg final product.

## 6.3 Enzyme Residues in the Final Food

The enzyme is added during the processing of snack type foods such as fabricated potato chips, and wheat dough based products such as cookies, crackers and bread. In dough based products, the enzyme is added to the dough before baking. In the fabricated potato chip application the enzyme is also added prior to a heat treatment or cooking step. The enzyme will be inactivated during these types of heat processing or cooking steps.



## 7. SAFETY EVALUATION

### 7.1 Safety of the Production Strain

The safety of the production organism must be the prime consideration in assessing the probable degree of safety of an enzyme preparation intended for use in food (Ref. 2,3). If the organism is nontoxic and nonpathogenic, then it is assumed that food or food ingredients produced from the organism, using current Good Manufacturing Practices, is safe to consume (Ref. 4). Pariza and Foster (1983) define a nontoxic organism as "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure" and a nonpathogenic organism as "one that is very unlikely to produce disease under ordinary circumstances". *A. oryzae* meets these criteria for nontoxigenicity and nonpathogenicity. In addition, *A. oryzae* is not considered pathogenic by JECFA (Ref. 27).

Barbesgaard et al. reviewed the safety of *A. oryzae* and describe it as having a very long history of safe industrial use, being widely distributed in nature, and being commonly used for production of food grade enzymes (Ref. 28; Appendix 3). *A. oryzae* is accepted as a constituent of foods (Ref. 27). *A. oryzae* has been used to produce soy sauce in the United States since before 1958 (Ref. 4, 28). Therefore, food ingredients from *A. oryzae* meet the criterion of "common use in foods in the US before 1958" and can be considered "generally recognized as safe", GRAS (Ref. 4). A GRAS petition, 3G0016, proposing affirmation that enzyme preparations from *A. oryzae* are GRAS for use in food was submitted to FDA and accepted for filing in 1973 (Ref. 29). Since that time, Novozymes and other companies have marketed enzyme preparations from *A. oryzae* in the US as GRAS substances. Carbohydrase, protease and lipase enzyme preparations from *A. oryzae* are now the subject of GRAS notices No. 90 and 113. Therefore, enzyme preparations from *A. oryzae* are also considered GRAS (Ref. 3,4,27).

An evaluation of the genetically modified production microorganism for the asparaginase, embodying the concepts initially outlined by Pariza and Foster, 1983 and further developed by IFBC in 1990, the EU SCF in 1991, the OECD in 1992, ILSI Europe Novel Food Task Force in 1996, FAO/WHO in 1996, JECFA in 1998 and Pariza and Johnson, 2001 demonstrates the safety of this genetically modified production microorganism strain. The components of this evaluation: the identity of the host strain, a description of the incorporated DNA, the sources and functions of the introduced genetic material, an outline of the genetic construction of the production strain, and some characteristics of the production strain and the enzyme derived from it are given in Section 2.

Because the genetic modifications are well characterized and specific, and the incorporated DNA does not encode and express any known harmful or toxic substances, the enzyme preparation derived from the genetically modified *A. oryzae* is considered safe (Ref. 4, 30).



### 7.1.1 Safe Strain Lineage

The safety of this *A. oryzae* production strain was established following published criteria for the assessment of the safe use of microorganisms used in the manufacture of food ingredients (Ref. 2, 4). The host strain, *A. oryzae* BECh2, has been thoroughly characterized as shown in sections 2.2. The introduced DNA is well-known and characterized in section 2.3. The procedures used to modify the host organism are well defined and commonly used. Therefore, the elements needed to establish a safe strain lineage as defined in Pariza and Johnson, 2001 (Ref. 2) have been met.

Novozymes' used the decision tree in Pariza and Johnson 2001 (Ref.2) as a basis for our safety assessment. The production strain is genetically modified by rDNA techniques as discussed in section 2. The expressed enzyme product is an asparaginase (See section 7.3). The enzyme preparation is free of transferable antibiotic resistance gene DNA. The introduced DNA is well characterized and safe for the construction of microorganisms to be used in the production of food grade products. The DNA is randomly integrated into the chromosome, however, the production strain is well characterized by qualified scientists and technicians. Novozymes has extensive experience working with *A. oryzae* production strains and has developed expertise in identifying and characterizing these strains in order to prevent contamination and ensure continuing acceptable, economic yields of a functional enzyme product. Research scientists, fermentation engineers, chemical operators, and quality control technicians follow standard aseptic microbiological procedures as well as specific Novozymes protocols for monitoring the biological activity, growth, and physiological characteristics of the production organism during strain improvement programs and during large scale industrial fermentations. In addition, the final commercial enzyme product must perform reproducibly, meet Novozymes' technical service department requirements, and consistently meet the needs of customers in the food industry. All of these periodic and continuous monitoring activities serve not only to guarantee customer satisfaction with Novozymes' enzyme products but also indicate that no unexpected secondary effects of the genetic modifications have been observed.

Finally, the production strain is derived from a safe lineage. Novozymes has used *A. oryzae* production strains for over 30 years. Table 2 below outlines some of Novozymes' products produced by *A. oryzae* production strains and the safety studies conducted on those products. We have published safety studies on two products produced from *A. oryzae* strains developed from *A. oryzae* A1560 (Ref. 31, 32). We have unpublished data on several other products produced by *A. oryzae*.

The recipient strain, *A. oryzae* BECh2, for this asparaginase is the same strain that was used in the construction of Novozymes' production strains for a modified lipase, glucose oxidase, and phospholipase which are the subjects of GRN Nos. 103, 106, and 142, respectively. Safety studies including 13 week oral toxicity in rats, Ames

test, and human lymphocyte cytogenetic assay, were performed on those preparations. Except for the coding sequence for the enzyme itself, the DNA that was introduced into the recipient strain to construct the asparaginase production strain is essentially the same as the introduced DNA in the GRN 103 production strain. Novozymes also has unpublished toxicological data on a phytase preparation and a xylanase preparation produced by an *A. oryzae* strain constructed from *A. oryzae* BECh2. All of these studies concluded that the test preparations did not exhibit any toxic or mutagenic effect under the conditions of the test. These studies support the view, that strains derived from *A. oryzae* BECh2 can be used safely for the production of food enzymes. The same well known procedures have been used to construct this *A. oryzae* production strain. Therefore, we would not expect to see any negative results in similar toxicological studies.

Table 2. Novozymes' products derived from *A.oryzae* strains

Enzyme	IUB no	Host strain <sup>1</sup>	Donor strain	Safety studies <sup>2</sup>	Published Safety Studies
Triacylglycerol lipase (GRN No. 43)	3.1.1.3	<i>Aspergillus oryzae</i> (A 1560)	<i>Humicola (now Thermomyces) lanuginosa</i>	Yes	Yes (Ref. 31)
Mucorpepsin (GRN No. 34)	3.4.23.23	<i>Aspergillus oryzae</i> (A 1560)	<i>Rhizomucor miehei</i>	Yes	No
Triacylglycerol lipase (GRASP 7G0323)	3.1.1.3	<i>Aspergillus oryzae</i> (A 1560)	<i>Rhizomucor miehei</i>	Yes	Yes (Ref. 32)
Triacylglycerol lipase (GRN No. 75)	3.1.1.3	<i>Aspergillus oryzae</i> (JaL 228)	<i>Fusarium oxysporum</i>	Yes	No
6-phytase	3.1.1.26	<i>Aspergillus oryzae</i> (JaL 228)	<i>Peniophora lycii</i>	Yes	No
Xylanase	3.2.1.8	<i>Aspergillus oryzae</i> (BECh2)	<i>Thermomyces lanuginosus</i>	Yes	No
Glucose oxidase (GRN No. 106)	1.1.3.4	<i>Aspergillus oryzae</i> (BECh2)	<i>Aspergillus niger</i>	Yes	No
Triacylglycerol lipase (GRN No. 103)	3.1.1.3	<i>Aspergillus oryzae</i> (BECh2)	<i>Thermomyces lanuginosus/ Fusarium oxysporum</i>	Yes	No
Phospholipase (GRN No. 142)	3.1.1.32	<i>Aspergillus oryzae</i> (BECh2)	<i>Fusarium venenatum</i>	Yes	No

<sup>1</sup> Development of the host strain, *A. oryzae* BECh2, from the wild type:



A. oryzae (A 1560)	wild type strain
A. oryzae (JaL 228)	alp-, amy-, Npl-
A. oryzae (BECh 2)	alp-, amy-, Npl-, CPA-, KA deficient

## Gene deletions:

Alp- : alkaline protease

Amy- : alpha-amylase

Npl- : neutral metallo protease I

CPA- : Cycloplazonic acid

## Gene mutation:

KA- : Kojic acid

<sup>2</sup>At least the following: 13 week acute oral toxicity in rats; Ames test; In vitro human lymphocyte cytogenetic assay

For this asparaginase, we have conducted an In vitro cytotoxicity test and an Ames test (as discussed in section 7.5). The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study.

Novozymes has repeatedly used the procedures outlined by Pariza and Johnson 2001 (Ref. 2) to evaluate the enzymes derived from *A. oryzae* production strains. The evaluations of three of the enzymes from the same host strain, BECh2, as that used in the construction of the production strain for this asparaginase enzyme have been documented in GRAS notifications to the FDA. Therefore, following the evaluation outlined in this section this production strain is considered to be derived from a safe lineage and is safe for use in the production of enzyme preparations for use in food.

### 7.1.2 Secondary Metabolites

*A. oryzae* is known to have the potential to produce the secondary metabolites, cycloplazonic acid (CPA), 3 ( $\beta$ )-nitropropionic acid (NPA) and kojic acid (KA). Novozymes' has shown that the whole aflatoxin gene cluster and the genes involved in CPA biosynthesis have been deleted from this host strain, *A. oryzae* BECh2.

One batch of asparaginase was analyzed for the secondary metabolites NPA and KA. The results are shown in Table 1.

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Table 1. Secondary Metabolite Analyses

	Unit	PPV 24743
Kojic Acid	mg/kg	ND (<1.4)
3-Nitropropionic acid	mg/kg	ND (<0.6)

ND = Not Detected (limit of detection is given in the brackets).

Based on information presented in this section as well as safety assessments conducted on KA, and NPA (Ref. 33, 34), it is concluded that the production strain, *A. oryzae* pCaHj621/BECh2#10, does not produce secondary metabolites of toxicological concern to humans.

## 7.2 Safety of the Donor Organism

The donor for the asparaginase enzyme is *Aspergillus oryzae*. See section 7.1 on the safety of *A. oryzae*.

## 7.3 Safety of the Asparaginase Enzyme

A wide variety of enzymes are used in food processing (Ref 2). Enzyme proteins do not generally raise safety concerns (Ref. 3, 35, 36). Exceptions could include enzymes that produce substances that are not ordinarily digested and metabolized or that produce toxic substances (Ref.37). Pariza and Foster (Ref. 3) note that very few toxic agents have enzymatic properties. As indicated in section 4, the subject of this GRAS notification is an asparaginase EC 3.5.1.1. Because aspariginases have been isolated from a variety of sources, animal cells, plant cells and microbial cells<sup>1</sup>, they are most likely part of the normal human diet. Asparaginases have been used in cancer research as anti-tumor agents over the last 20 years (Ref. 38). Some asparaginases have been used clinically in the treatment of leukemia especially in children (Ref. 38, 39, 40).

### 7.3.1 Consideration of the Allergenic Potential of the Enzyme

Enzymes are proteins and proteins can be potential allergens. However, only a small percentage of all dietary proteins are allergens. Novozymes is not aware of any allergic reactions caused by the ingestion of asparaginase. Novozymes' believes that, in general, the risk of allergenicity due to ingestion of enzymes is negligible based on the following considerations:

- the long history of safe use of a wide variety of enzymes in food and food processing
- the ubiquitous nature of enzymes

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- the extremely low exposure potential
- the purpose

As discussed in Section 7.3, enzyme proteins do not generally raise safety concerns. A wide variety of enzymes have been used safely for centuries in food processing. Enzymes are found in many cells and tissues of plants and animals including those that are consumed by man. They are ubiquitous and in this respect, common and ordinary. They are a normal part of the human diet.

Enzymes are a special type of protein with a highly specific catalytic function. They are essential to many biochemical reactions in microorganisms, plants, animals and humans. Due to the specific nature of enzymes, only very small amounts are required to achieve the desired effect in food.

Based on the fact that:

- there are no known cases of allergic responses to asparaginase in food,
- known cases of allergic responses to food enzymes are very rare, and
- exposure to this enzyme protein is extremely low,

the allergy risk due to the ingestion of this asparaginase is negligible.

#### 7.4 Safety of the Manufacturing Process

The asparaginase meets the purity specifications for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex. As described in Section 3, the asparaginase preparation is produced in accordance with current good manufacturing practices, using ingredients that are acceptable for general use in foods, and under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for production of microbial enzymes (Ref. 18-20).

#### 7.5 Safety studies

This section describes the studies and analysis performed to evaluate the safety of the use of the asparaginase.

##### 7.5.1 Description of Test Material

The safety studies described below were conducted on a liquid asparaginase enzyme concentrate that was prepared according to the description given in Section 3 except that stabilization and standardization were omitted.

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## 7.5.2 Studies

The following studies were performed:

- Test for mutagenic activity (Ames test )
- In vitro* Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture

These tests are described in Appendix 4. The conclusion of these tests is that the test preparation is considered non-cytotoxic and does not induce gene mutations in bacteria under the conditions of the study.

## 7.6 Estimates of Human Consumption and Safety Margin

The enzyme is largely heat inactivated during the food manufacturing processes in which it is applied. However, in order to illustrate a "worst case" situation the following calculations are made assuming that all enzyme activity is retained in the final product.

Acrylaway has an activity of 3500 ASNU/g and an approximate content of 4 % TOS (Total Organic Substances from the fermentation, mainly protein and carbohydrate components).

### 7.6.1 Estimates of human consumption

Acrylaway is to be used for acrylamide reduction in various food applications in dosages up to a maximum of 2500 ASNU per kg processed food, corresponding to 70 g of Acrylaway per 100 kg processed food.

Because the enzyme may be used for a variety of food applications, the estimation of human consumption is based on the Budget method (Ref. 41; Appendix 5).

In order to demonstrate a worst case calculation, an exaggerated human intake is estimated using the following assumptions.

- It is assumed that all processed foods are produced using Acrylaway as a processing aid, used at the highest recommended dosage.
- According to the Budget method, a conservative estimate for the food intake is 25 g per kg body weight per day of which processed food is 50% of the food intake or 12.5 g per kg body weight per day.
- The calculation is made assuming that all TOS remains in the final product. Acrylaway contains 4 % TOS.

The maximum recommended dosage is 70 g of Acrylaway per 100 kg processed food. Acrylaway contains 4 % TOS, giving an overestimate of 28 mg TOS/kg processed food.

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Based on the estimate for processed food intake of 12.5 g per kg body weight per day, the intake of Acrylaway corresponds to  $28 \times 0.0125 = 0.35$  mg TOS per kg body weight per day.

#### 7.6.2 Safety margin

The safety margin is calculated as dose level with no adverse effect (NOAEL) divided by the estimated human consumption. The NOAEL dose level in the 13 weeks oral toxicity studies in rats for similar enzyme preparations from *A. oryzae* BECh2 strains was the highest dosages possible, approximately 1000 mg TOS/kg/day.

The estimated human consumption is 0.35 mg TOS/kg/day

The safety margin can thus be calculated to be  $1000/0.35$  or approximately 2500.

**On this basis it is concluded that Acrylaway is safe for its intended use in the food industry.**

#### 7.7 Results and Conclusion

On the basis of the evaluation contained in Section 7, a review of the published literature, the history of use of *A. oryzae*, and the limited and well defined nature of the genetic modifications, the asparaginase enzyme preparation can be safely manufactured and used as a processing aid to reduce acrylamide levels in food.

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Appendix 2

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# Asparaginase Technology Sheet



## Introduction to acrylamide

In 2002, the Swedish National Food Authorities and the University of Stockholm discovered considerable levels of acrylamide in starch-based foods processed or cooked at high temperatures. Acrylamide is classified as "probably carcinogenic to humans" by the International Agency for Research on Cancer and considered to be a reproductive toxin.

Foods	JECFA report*	Swedish study**
French fries	16-30%	12%
Potato chips	6-46%	—
Coffee	13-39%	54%
Cookies	10-20%	—
Bread	10-30%	—
Crisp bread	—	9%

\*JECFA report: 64th meeting, Rome, Feb. 8-17, 2005.  
\*\*Karolinska Institute & Harvard School of Public Health.

Acrylamide levels found in common foods

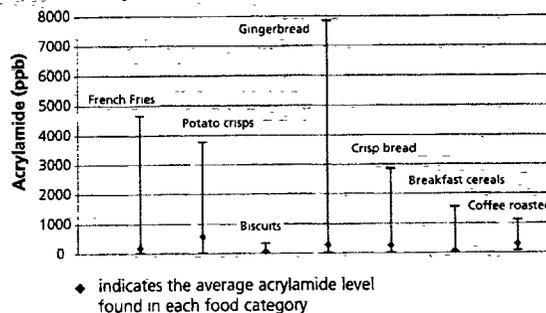


Table 1. Results from two studies showing estimated contributions of specific foods to total daily dietary acrylamide exposure. Acrylamide intake varies by region according to prevailing diets. The international mean intake of acrylamide is estimated to be 1 microg/kg bodyweight/day

Figure 1. Acrylamide amounts (ppb) in common foods.

Source: [http://www.imm.jrc.be/html/activities/acrylamide/EUacrylamidelevelmonitoringdatabase\\_status/june2005.xls](http://www.imm.jrc.be/html/activities/acrylamide/EUacrylamidelevelmonitoringdatabase_status/june2005.xls)

## Acrylamide formation and enzymatic reduction with asparaginase

The main mechanism for acrylamide formation in starchy foods involves reducing sugars and the amino acid asparagine, both common to these foods. The sugars react with asparagine when the food is heated and, through a cascade of reactions, the side chain of asparagine is converted to acrylamide.

One potentially very effective means of reducing acrylamide formation is through the removal of asparagine from starchy foods with the enzyme asparaginase, which converts asparagine to another common amino acid, aspartic acid.

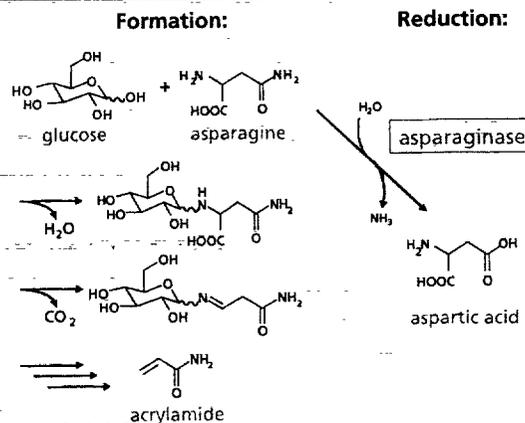


Figure 2 Mechanism for acrylamide formation and enzyme action.



Unlocking the magic of nature

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# Asparaginase Technology Sheet

## Application examples of Novozymes asparaginase in lab scale

Various food products were tested in the laboratory for acrylamide reduction using Novozymes' asparaginase.

## Summary

Food product	Acrylamide reduction (%)
Semi-sweet biscuits	80-85%
Fabricated potato chips	80-98%
Crisp Bread	84-92%
Ginger nut biscuits	64-79%
Toast bread	~40%
French fries	80% vs a Control 50-60% vs a Blank

Table 2 Acrylamide reductions achieved with Novozymes' asparaginase in a variety of food products.

## Crisp bread

Figure 3 illustrates the influence of dough temperature on enzyme activity in crisp bread. Dough treated with asparaginase showed an acrylamide reduction of 84-92%, even at low temperatures.

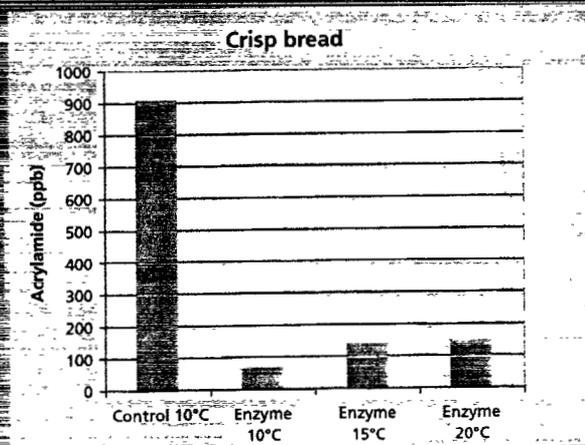


Figure 3. Acrylamide level (ppb) in crisp bread resulting from enzyme treatment at various dough temperatures

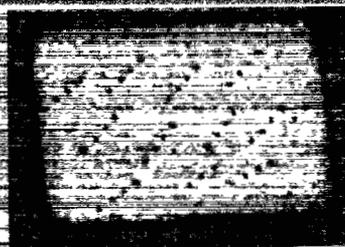


Figure 4. Crisp bread prepared from asparaginase treated dough.

# Asparaginase Technology Sheet

## Semi-sweet biscuits

The influence of enzyme dosage and holding time on the acrylamide levels in semi-sweet biscuits is shown in Figure 5.

Acrylamide was reduced (up to ~90%) with increasing dosage and longer holding times. No differences were noted in sensory analysis.

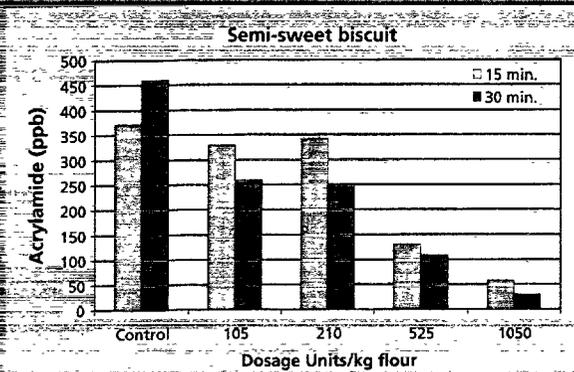


Figure 5. Acrylamide reduction (ppb) in semi-sweet biscuits.

Control                      Enzyme-treated

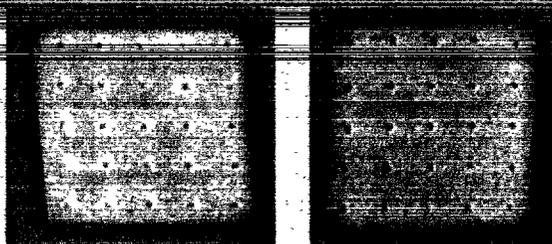


Figure 6. Semi-sweet biscuits prepared from control and enzyme treated dough.

## Toast bread

The influence of Novozymes' asparaginase on toast bread is shown in Figure 7. Pan bread was prepared according to the sponge and dough method, with enzyme added directly to the dough. Bread slices were toasted, ground and then sent for analysis. A reduction of 38% was achieved.

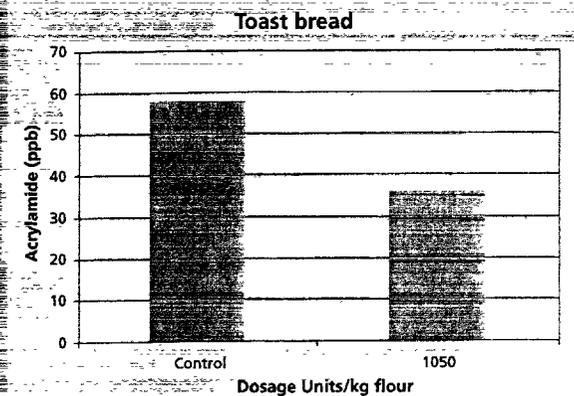


Figure 7. Acrylamide levels (ppb) in toast bread.

Control                      Enzyme-treated



Figure 8. Toast bread prepared from control and enzyme treated dough.

# Asparaginase Technology Sheet

## Fabricated potato chips

Figure 9 illustrates the influence of varying dosages of Novozymes' asparaginase on acrylamide levels (ppb) in fabricated potato chips. An acrylamide reduction of 98% was achieved with the highest dosage.

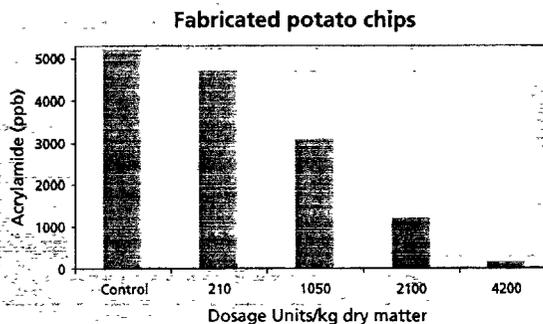


Figure 9. Acrylamide levels (ppb) in fabricated potato chips treated with varying dosages of Novozymes' asparaginase.

## French fries

Figure 10 illustrates the reduction in acrylamide levels (ppb) in french fries treated with water only and with water plus Novozymes' asparaginase. Control samples had no treatment. Different batches of potatoes (represented by the different colors in Figure 10) were used to illustrate the large variation of acrylamide levels found in these potatoes. Irrespective of the potato batch, enzyme treatment consistently reduced acrylamide levels by approximately 85% compared to the control. Water soaking alone was less efficient, reducing acrylamide levels by approximately 60%.

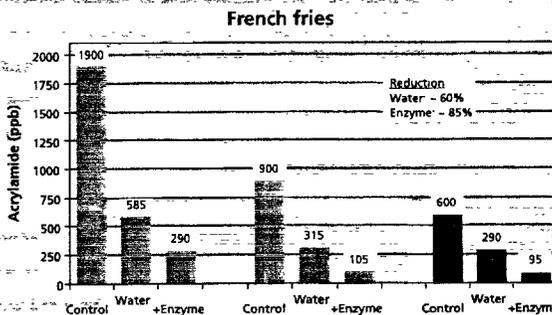


Figure 10. Reduction in acrylamide levels (ppb) in french fries treated with water only (~60%) and with water plus Novozymes' asparaginase (~85%).



Figure 11. French fries prepared from enzyme treated potato strips.

For further information on Novozymes' asparaginase, contact Ms. Anett Lund-Nielsen, Tel. +41 61 76 56 111, [aln@novozymes.com](mailto:aln@novozymes.com)



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Appendix 4

Safety & Toxicology

Date: 19. April 2006  
File: 2006-17976-01  
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## FINAL REPORT

Asparaginase, batch PPV 24743  
*In Vitro* Cytotoxicity Test:  
Neutral Red Uptake in L929 Monolayer Culture

Study No.: 20068045

*Author:*  
Jens Lichtenberg

*Issued by:*  
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## GLP – COMPLIANCE STATEMENT

**REPORT :** Asparaginase, batch PPV 24743.  
*In Vitro* Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture.

**STUDY No.:** 20068045

The sample of Asparaginase, batch PPV 24743 was received from Recovery Pilot Plant, Novozymes A/S.

This study was conducted at the department of Safety & Toxicology, Novozymes A/S in compliance with the following current Good Laboratory practice Regulations:

OECD Principles of GLP, ENV/MC/CHEM (98) 17, 1998.

Date: 20 April 2006

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Jens Lichtenberg  
Study Director

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## QUALITY ASSURANCE STATEMENT

Report: Asparaginase, batch PPV 24743  
In Vitro Cytotoxicity Test:  
Neutral Red Uptake in L929 Monolayer Culture

STUDY NUMBER 20068045

The conduct of this study has been subject to appropriate inspections and the report has been reviewed according to the relevant Standard Operation Procedures of Novozymes A/S Quality Assurance.

Inspection/Audit	Dates of inspection	Dates of Audit Report signed by Study Director	Dates of Audit Report signed by Study Management
Protocol	22 mar 2006	27 mar 2006	3 apr 2006
Reading and calculation	29 mar 2006	7 apr 2006	7 apr 2006
Report	18 apr 2006	19 apr 2006	19 apr 2006

*20 April 2006*

Date

\_\_\_\_\_  
Annie Christensen  
Quality Assurance

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1. GENERAL INFORMATION

**STUDY** Asparaginase, batch PPV 24743  
*In Vitro* Cytotoxicity Test: Neutral Red Uptake in L929 Monolayer Culture.  
Study No. 20068045

**STUDY DIRECTOR** Jens Lichtenberg, DVM  
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**TEST FACILITIES** Safety & Toxicology  
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**ARCHIVE** QM Central Archive  
Novozymes A/S  
Krogshøjvej 36  
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**DATES OF STUDY** Study Initiation Date: 17 March 2006  
Experimental Starting Date: 27 March 2006  
Experimental Completion Date: 29 March 2006

**PERSONNEL INVOLVED IN THE STUDY**

**TECHNICIANS** Pia Schock Kristensen - Safety & Toxicology

**DATE OF FINAL REPORT**

Date: 20 April 2006

Jens Lichtenberg  
Safety & Toxicology

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## 2. SUMMARY

Asparaginase, batch PPV 24743, was examined for cytotoxic potential in an *in vitro* bioassay, the Neutral Red Uptake assay, using L929 mouse fibroblast cells grown as monolayer culture in 96-well microplates.

The time of exposure for the test substance and positive control was 24 hours. The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU<sub>50</sub>).

The NRU<sub>50</sub> value for Asparaginase, batch PPV 24743, was estimated to be >30 mg/mL. This result indicates that Asparaginase, batch PPV 24743, is non-cytotoxic, in the present *in vitro* Neutral Red Uptake assay applying the mouse fibroblast cell line L929 as test system.

## 3. INTRODUCTION

The neutral red uptake (NRU) assay is a quantitative, colorimetric method to measure the cell viability. The assay procedure is a cell survival/viability assay based on the ability of viable cells to incorporate and bind Neutral Red (NR), a supravital dye. NR is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion and accumulates intracellularly in lysosomes. Alteration of the cell surface or the sensitive lysosome membrane leads to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics (test substance) result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable and damaged or dead cells, and this is the basis of the assay.

The test system L929 is an established mouse fibroblast cell line. It was selected for the ease with which these cells are maintained and grown as monolayer culture and it is commonly used as first order test system for general cytotoxicity.

The L929 cells, when appropriately maintained in culture in a sub confluent state, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in cell death and/or a reduction of the growth rate as reflected by cell number. The amount of neutral red uptake by the cells after exposure to the test substance is an indication of the toxicity of the test article.

Cytotoxicity of test substance is expressed as the concentration of test material required to reduce the uptake of NR to 50% of untreated control cells after 24 hours of exposure.

## 4. MATERIALS

### 4.1. Media and reagents

- The growth medium Earle's Minimum Essential (EMEM) was supplemented with Fetal Bovine Serum (FBS), NaHCO<sub>3</sub> and antibiotics/ antimycotics. The medium was stored at 4°C for not more than a month. Prior to use the medium was allowed to obtain room temperature.

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- 0.05% Trypsin/EDTA solution was thawed and stored at 4°C for not more than a month. The solution was allowed to obtain room temperature prior to use.
- Neutral Red (NR) stock solution was made by dissolving 5 mg NR dye pr. mL Milli-Q water. The solution was sterilized by filtration and stored at room temperature for max. 6 months.
- NR Medium was made by dissolving 500 µL NR stock solution in 50 mL EMEM medium.  
The NR medium was incubated overnight at 37°C and centrifuged at 1100 x g for 5 min before use. The solution was sterilized by filtration before use.
- NR Desorb solution was prepared from glacial acetic acid 1 % v/v, ethanol 50 % v/v and Milli-Q water 49 % v/v. The NR desorb solution was stored in a tightly closed bottle at room temperature for no longer than 3 months.

#### 4.2 Test substance

The test substance, Asparaginase, batch PPV 24743, a brown liquid at room temperature, was received from Recovery Pilot Plant on 10 October 2005 and immediately stored in a freezer.

Enzyme activity: 4440 ASNU/g  
 Specific gravity: 1.049 g/mL  
 TOS (Total Organic Solids): 8.4 % w/w

A stock solution of 30 mg/mL was prepared in growth medium the day before being applied to the test system. The stock solution was stored at 4°C until dilution and use. Stability data from NZ Study No. 20058010 (Characterization-report of tox-batch, Asparaginase, batch PPV 24743, Luna No. 2005-09224-01/KM) demonstrates that the test material is stable for at least 24 hours at 4 °C. Osmolality and pH of the stock solution was measured to be 315 mOsm/kg and pH 7.0, respectively, which were within acceptable ranges.

#### 4.3 The positive control

The Sodium Dodecyl Sulphate (SDS) stock solution 1200 µg/mL, was diluted in growth medium to obtain concentrations of 120, 100, and 80 µg/mL and used as a positive control substance. The stock solution was sterilized by filtration and stored at 4°C for no more than 12 months. The solution should obtain room temperature prior to use.

#### 4.4 Test system

The Neutral Red Uptake Assay in the present study is performed according to the principles given in *INVITTOX* protocol No. 46, "BALB/C 3T3 Cytotoxicity Test". *INVITTOX*, a database on test method protocols created in 1989, was originally based at the Fund for the Replacement of Animals in Medical Experiments (FRAME, UK) and sponsored by the European Commission and the European Research Group for Alternatives Toxicity Testing (ERGATT). With the establishment of SIS, Scientific Information Service, in 1996, the responsibility for *INVITTOX* has been taken over by ECVAM, the European Centre for the Validation of Alternative methods.

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The present test system is an established mouse fibroblast cell line L929. It was received from Imperial Laboratories, UK, on the 8 September 1999, lot number 057777, identified as passage No 567. The cells were shipped in liquid medium and immediately grown in Ham's growth medium from the supplier. Thereafter the cells were grown in EMEM with 10% FBS at 37°C and 5% CO<sub>2</sub>. When the culture was confluent it was trypsinated and the cells were stored in liquid N<sub>2</sub> as a first NZ subculture cell master. To decrease the temperature at which ice is formed, 8-10% DMSO was added as cryoprotective agent.

From the first subculture master the first passage use-culture was made.

After approximately 20 passages, a new use-culture from the freezer was taken into use. The cells were thawed rapidly in a water bath at 37°C and put into growth medium. The DMSO is removed by centrifuging for 5 min at 200 g and the deposit of cells was suspended in medium again. The cell suspension was then incubated in a culture flash at 37°C and 5% CO<sub>2</sub>.

The present cell culture was passage number 574, i.e 7 passages after the cell culture was received from the supplier.

## 5. METHODS

The test was performed according to standard method SAT-SM-0010.01, version 05. L929 fibroblasts were grown in EMEM medium with 10% FBS in 96-well micro-plates, 150 µL (5 x 10<sup>5</sup> cells per mL) cell culture was added per well. Plates were incubated for 24 hours at 37°C, establishing a near confluent monolayer. 150 µL test material dilutions in medium were added (at least 4 replicates) and incubated for 24 hours at 37°C. The cell morphology was checked before and after addition of test material. One extra 96-well plate was used to determine the cell viability at time= 0, before the addition of the test material, using the Neutral Red assay as described below.

The test material was replaced by 150 µL Neutral Red solution. Plates were in the CO<sub>2</sub> incubator for 3 hours at 37°C, thereafter washed twice in medium without FBS, before 100 µL Neutral Red desorb solution was added to each well to leach the stain from the cells. The plates were agitated well to evenly distribute the released Neutral Red, and the absorbance at 540 nm (OD<sub>540</sub>) of each well was measured to indicate the number of cells surviving exposure to the test materials. The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU<sub>50</sub>) by extrapolation from the dose-response curve.

Dosing:

The following concentrations were selected for the Asparaginase, batch PPV 24743, in the present test model:

30, 10, 3, 1 and 0.3 mg/mL per mL growth medium (EMEM, 10% FBS).

Positive control: 120, 100, 80 µg SDS per mL growth medium.

Appropriate solvent control wells (untreated control cells) and blank controls (solvent alone without cells) were included on the micro-plate.

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## 6. RESULTS AND DISCUSSION

The basis of the present test system is that the uptake of Neutral Red into the lysosomes of living cells is a good end-point of cytoplasmic membrane integrity. The test can be used as a rapid screening bioassay in a first order *in vitro* battery which can provide an indication of toxicity, related to the concentration of the test substance.

The results obtained in this investigation appear from table 1 and Fig 1.

Table 1:

Test substance	Concentration mg/mL	Viability %	NRU <sub>50</sub> mg/mL
Asparaginase, batch PPV 24743	0	100	>30
	0.3	102	
	1	103	
	3	103	
	10	95	
	30	83	
Positive control	Concentration µg/mL	Viability %	NRU <sub>50</sub> µg/mL
SDS	0	100	93
	80	84	
	100	20	
	120	1	

The cell viability of untreated control cells in medium at time 0 and 24 hrs. was estimated, showing that the number of control L929 cells had increased 2.7 x over the exposure period, thereby confirming that the cells were in growth phase. The average doubling time for fibroblasts in cell culture flasks is known to be approx. 24 hrs.

The concentration of the test substance required to reduce the viability of the treated test system to 50% of that of the untreated control test system was determined as the endpoint (NRU<sub>50</sub>) by extrapolation from the dose-response curve.

The estimated NRU<sub>50</sub> values for Asparaginase, batch PPV 24743, and the positive control, SDS, are given in table 1.

However, due to the low toxicity of Asparaginase, batch PPV 24743, a clear NRU<sub>50</sub> value could not be deduced from the present data. The NRU<sub>50</sub> value for Asparaginase, batch PPV 24743 is above a test concentration of 30 mg/ml.

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Fig.1

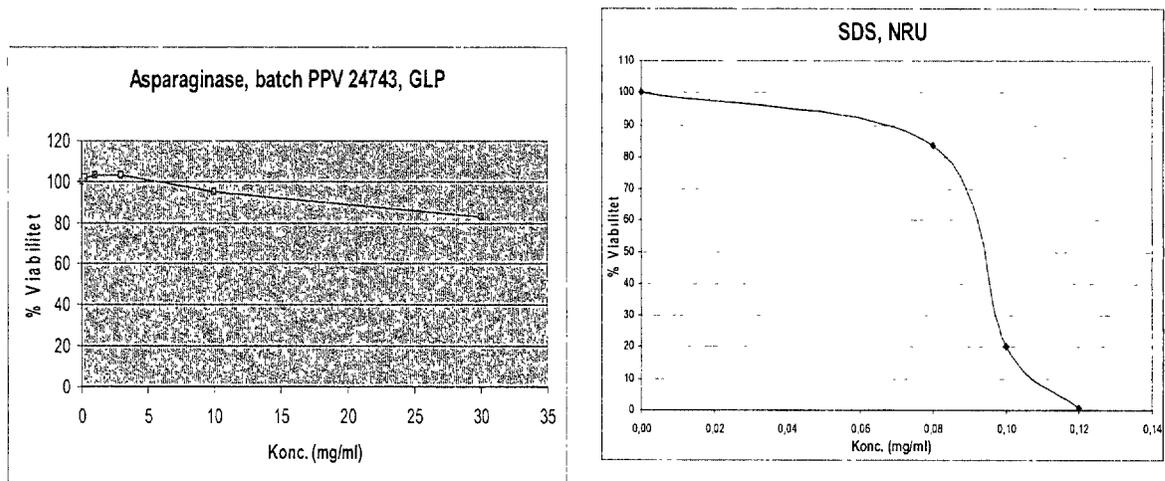


Fig 1. The concentration-response cytotoxicity curves for Asparaginase, batch PPV 24743 and SDS, used, if feasible, to estimate the mid-point cytotoxicity value,  $NRU_{50}$ .

The positive control, SDS, gave a  $NRU_{50}$  value of 93  $\mu\text{g}/\text{mL}$ . The acceptance criteria is set to be within 2 standard deviations of the historical mean ( $97 \pm 6.0\mu\text{g}/\text{mL}$ ) thereby meeting the acceptance criteria of a valid test. The estimated  $NRU_{50}$  value of SDS is fully in line with the results given in the reference, *INVITTOX* protocol No. 46.

## 7. CONCLUSION

The results show, that Asparaginase, batch PPV 24743 is non-cytotoxic exhibiting a  $NRU_{50}$  value above a test concentration of 30 mg/ml, in the present *in vitro* Neutral Red Uptake assay applying the mouse fibroblast cell line L929 as test system.

## 8. DATA STORAGE

Report, protocol and the raw data or exact copies thereof are filed in the QM Central Archive, Bagsvaerd.

## 9. REFERENCES

(1) *INVITTOX* protocol No. 46, "BALB/C 3T3 Cytotoxicity Test", Jan. 1992 (available electronically from SIS, Scientific Information Service, at ECVAM's website)

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Safety & Toxicology

Date: 18.Maj.2006  
Proj: DEV 00599  
File: 2006-20941-01  
Ref.: UF/PBjP

## REPORT

**Asparaginase, PPV 24743: Test for Mutagenic Activity  
with Strains of *Salmonella typhimurium* and *Escherichia coli*.**

Study No. 20068039

*Author :*  
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## GLP - COMPLIANCE STATEMENT

**REPORT :** Asparaginase, PPV 24743: Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli* .

**STUDY No.:** 20068039

A sample of Asparaginase Batch Number: PPV 24743 was received from Recovery Pilot Plant, Novozymes A/S.

This study was conducted at the department of Safety & Toxicology, Novozymes A/S in compliance with the following current Good Laboratory Practice Regulations:

OECD, ENV/MC/CHEM(98)17, 1998

Date : 29 May 2006

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Peder Bjarne Pedersen  
Study Director

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# QUALITY ASSURANCE STATEMENT

Report. Asparaginase, PPV 24743:  
Test for mutagenic activity with Strains of Salmonella  
typhimurium and Escherichia coli

STUDY NUMBER 20068039

The conduct of this study has been subject to appropriate inspections and the report has been reviewed according to the relevant Standard Operation Procedures of Novozymes A/S Quality Assurance..

Inspection/Audit	Dates of inspection	Dates of Audit Report signed by Study Director	Dates of Audit Report signed by Study Management
Protocol	10 mar 2006	18 maj 2006	19 maj 2006
Pure plate	24 mar 2006	28 mar 2006	28 mar 2006
Report	24 maj 2006	29 maj 2006	29 maj 2006

29 May 2006  
Date

.....  
Annie Christensen  
Quality Assurance

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## 1. GENERAL INFORMATION

**STUDY** Asparaginase, PPV 24743: Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli*.  
Study No. 20068039

**STUDY DIRECTOR** Peder Bjarne Pedersen  
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**TEST FACILITIES** Safety & Toxicology  
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**ARCHIVE** QM Central Archive  
Novozymes A/S  
Krogshøjvej 36  
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**DATES OF STUDY**  
Study initiation date: 07. Mar. 2006  
Experimental start date: 13. Mar. 2006  
Experimental termination: 05. May. 2006

### PERSONNEL INVOLVED IN THE STUDY

**TECHNICIANS** Ulla Festersen (UF) - Safety & Toxicology  
Jytte Nordlund Petersen (JNP), - Safety & Toxicology  
Pia Schock Kristensen (PScK) - Safety & Toxicology

### DATE OF FINAL REPORT

Date: 29 May 2006

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Peder B. Pedersen  
Safety & Toxicology

## 2. SUMMARY

Asparaginase (Batch Number: PPV 24743) was examined for mutagenic activity in the bacterial reverse mutation assay using *Salmonella typhimurium* strain TA1535, TA100, TA1537, TA98 and *Escherichia coli* WP2uvrA.

The study was conducted with and without the metabolic activation system S9 - a liver preparation from male rats, pre-treated with Aroclor 1254, and the co-factors required for mixed function oxidase activity (S9 mix).

Crude enzyme preparations, like the present Asparaginase, contain the free amino acid L-histidine, most often in an amount, which exceeds the critical concentration for incorporation in the direct standard assay.

To overcome this problem all *Salmonella* strains were exposed to Asparaginase in liquid culture ("treat and plate assay"). Bacteria were exposed to 6 doses of the test substance in a phosphate buffered nutrient broth for 3 hours with 5 mg (dry matter) per ml as highest concentration. After incubation the test substance was removed by centrifugation prior to plating.

Usually the content of tryptophan in enzyme preparations is low and insignificant. Therefore the part of the study comprising *Escherichia coli* was initially conducted with the strain WP2uvrA using the direct plate incorporation assay. 6 doses of the test substance were applied with 5 mg (dry matter) per plate as the highest dose level followed by successive bi-sections between doses.

The results of this experiment showed that the Asparaginase test article significantly support growth of the tryptophan requiring *E.coli* strain by direct plate incorporation. The bacterial background lawn becomes increasingly dense and conspicuous with increasing doses of Asparaginase due to content of tryptophan. As a result, significant dose-related increases in the number of spontaneous revertants are observed in these two test series.

As a consequence, two new and independent experiments were conducted with the strain *E.coli* WP2uvrApKM101 applying the treat and plate assay.

Asparaginase is a fluid enzyme preparation. Besides amino acids it contains an abundance of various nutrients, and composes a rich growth medium to the test bacteria. In a treat and plate assay bacteria are exposed to different doses of the test substance in separate liquid cultures for a certain time. As a consequence variation in the number of spontaneous revertant colonies may occur due to growth stimulation/inhibition in the treat and plate assay.

These circumstances are reflected in the present study. No toxicity of the test substance to the bacteria is observed. On the contrary weak growth stimulation is evident to some extent in some of the test series demonstrated as increases in the viable count of exposed cultures compared to the solvent control. Concomitantly low numerical increases in the number of spontaneous revertant colonies are present as expected. These increases are hardly reproducible and are obviously not of biological importance.

In an attempt to demonstrate this relation between the viable count and revertant colony count an additional third experiment was carried out with the two *Salmonella* strains TA98 and TA100 and *E. coli* WP2uvrApKM101 including a re-growth experiment of all cultures exposed in the treat and plate assay. No numerical increases worth consideration were observed in any of these test series either before or after re-incubation.

We consider a test substance as positive in this treat and plate assay when it has induced at least a doubling in the mean number of revertants per plate compared to the appropriate solvent control in one or more of the strains, in the presence or absence of S9, if this response is dose related (at least 3 doses) and reproducible. If a numerical increases below a doubling is observed and if these increases is dose related and reproducible and not accompanied by significant increases in the viable bacterial count the result is considered as equivocal and need further clarification.

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No treatments of any of the *Salmonella* and *E.coli* strains with Asparaginase resulted in any increases in revertant numbers that meets these criteria for a positive or equivocal response.

It was concluded, that the results of the experiments, described in this report, give no indication of mutagenic activity of Asparaginase (Batch Number: PPV 24743) in the presence or absence of metabolic activation, when tested under the conditions employed in this study.

### 3. INTRODUCTION

Bacterial reverse mutation assays have been recognised and used for more than two decades as a rapid, sensitive and reliable method of evaluating the mutagenic potential of chemicals. Bacterial systems offer several advantages to other test systems. They can be grown in large numbers in a short time, enabling the detection of very rare mutational events. Further, extensive knowledge of bacterial genetics has allowed the construction of special strains, which are more sensitive than the wild-type strains to a variety of agents.

The reversion of bacteria from growth-dependence of a particular amino acid to be able to grow in the absence of that amino acid is the most widely used marker in reverse-mutation assays. The genetic target is small, specific and selective, and the phenotypic effect of the reverse mutation is easily detected.

A wide range of strains within the species *Salmonella typhimurium* (Ames strains) and *Escherichia coli* have been constructed in order to make the test system more sensitive and selective to different classes of chemical mutagens.

By incorporation of the post-mitochondrial supernatant (S9) from the livers of rats pre-treated with an enzyme inducer Aroclor 1254, the metabolising systems present in mammalian cells are mimicked to facilitate the detection of a wide range of pro-mutagens.

This report describes investigations performed to assess the activity of Asparaginase (Batch Number: PPY 24743) in amino acid dependent strains of *Salmonella typhimurium* and *Escherichia coli* capable of detecting both induced frame-shift (TA1537 and TA98) and base-pair substitution mutations (TA1535, TA100 and WP2uvrA).

Asparaginase is a microbial enzyme preparation derived from submerged pure culture fermentations of a non-pathogenic and non-toxic strain. It contains a variety of unspent medium residues, including low concentrations of free amino acids like histidine and tryptophan.

This complexity poses several problems during mutagenicity testing in vitro. In the Ames test it composes a rich growth medium to the test bacteria, resulting in completely different and poorly defined environments of exposed cultures compared to control cultures. The main problem is the content of utilizable histidine and sometimes also tryptophan in the test material, since the principle of the Ames test is the histidine auxotrophy of the *Salmonella* tester strains and tryptophan auxotrophy of the *E.coli* strains. Therefore *Salmonella* strains were exposed to Asparaginase in liquid culture ("treat and plate assay").

The part of the study comprising *Escherichia coli* was initially conducted with the strain WP2uvrA using the direct plate incorporation assay. However since the test article supported growth of the test bacteria in this procedure due to the content of tryptophan two new independent experiments were conducted with the strain *E.coli* WP2uvrApKM101 in the treat and plate assay with and without metabolic activation.

Finally, due to weak growth stimulation in the treat and plate assay, a third experiment with the *Salmonella* strains TA98 and TA100 and *E.coli* WP2uvrApKM101 were conducted applying the treat and plate assay and including a re-growth procedure.

The study was conducted in accordance with OECD Guideline for testing of chemicals, No. 471: Bacterial Reverse Mutation Assay" (July 1997 concerning the general specifications of the test. However the exposure of test bacteria in liquid culture ("treat and plate") is not specifically described in any guidelines.

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## 4. MATERIALS

### 4.1. Test substance

Asparaginase (Batch Number: PPV 24743) was received from Recovery Pilot Plant on the 1. December 2005, and immediately stored in a freezer. The substance was a brown liquid with a declared content of 10.5 % (w/w) dry matter.

A standard solution of 5% (w/v) dry matter was prepared in deionised water and sterilised by filtration.

Samples were sterilised by filtration and the sterility was confirmed by plate counting. Solution was stored at 4°C and used as test substance.

### 4.2. Positive control substances

Chemical	Source	Lot.No.
2-Nitrofluorene (2-NF)	Aldrich-Chemie	S 08447-155
9-Aminoacridine (9-AA)	SIGMA Chemical Company	106F-06682
N-Methyl- N'-Nitrosoguanidine (MNNG)	Aldrich-Chemie	15427 LO
N-Ethyl-N'-Nitro-N-Nitrosoguanidine (ENNG)	Aldrich-Chemie	08228 ES
2-Aminoanthracene (2-AA)	SIGMA Chemical Company	S 11804-492

All positive control substances were dissolved in dimethyl sulphoxide (spectrophotometric grade) obtained from Merck, Darmstadt, Germany.

### 4.3. Liver homogenate - S9

A commercial preparations of S9 from Aroclor 1254 induced Sprague Dawley rats was obtained from ICN Pharmaceuticals, Inc./Cappel Biochemical Division, 1263 S. Chillicothe Rd. Aurora, Ohio 44202. Specifications of the preparation, the enzymatic properties and metabolic activation from the supplier are archived as raw data.

The tubes with S9 were received frozen in dry ice and were immediately stored in a -80°C ultra low freezer at Safety & Toxicology, Novozymes.

### 4.4. Plates

As selective substrate for reverted bacteria Vogel-Bonner medium E agar plates with 2% glucose were prepared in-house as described in Appendix 3.

All plates were stored at 4°C in closed plastic bags and examined for contamination and dryness before use.

### 4.5. Bacteria

#### Salmonella typhimurium

Four strains of *Salmonella typhimurium* were used:

*S. typhimurium* TA1535

*S. typhimurium* TA100

*S. typhimurium* TA1537

*S. typhimurium* TA98

All these strains contain mutations in the histidine operon, thereby imposing a requirement for histidine in the growth medium. They all contain GC base-pairs at the site of the histidine mutation, and are therefore selective for agents which react predominantly with these bases. Three mutations in the histidine operon are involved:

his G 46 (TA1535 and TA100) is a missense mutation which is reverted to prototrophy by a variety of mutagens that cause base-pair substitutions.

his C 3076 (TA1537) contains a frame-shift which appears to have added a GC base-pair. This mutation is reverted for example by 9-Aminoacridine and epoxides of polycyclic hydrocarbon.

his D 3052 (TA98) also contains a frame-shift mutation with a sequence of repeated GC, which is reverted with the deletion of 2 of these base-pairs. It is readily reverted by aromatic amines and derivatives.

All 4 strains contain the deep rough (*rfa*) mutation, which deletes the polysaccharide side chain of the polysaccharide coat of the bacterial cell surface. This deletion increases cell permeability to more hydrophobic substances and, furthermore, greatly decreases the pathogenicity of these organisms.

The *uvrB* deletion renders the strains incapable of excision repair, making them more sensitive both to the mutagenic and lethal effects of a wide variety of mutagens (e.g. polyaromatic hydrocarbons), since the strains can not excise DNA adducts.

These 2 deletions include the nitrate reductase (*chl*) and biotin (*bio*) genes also.

Strain TA98 and TA100 are derived from strain TA1538 and TA1535 respectively by the addition of a plasmid, pKM101, which confers resistance to ampicillin. This plasmid also carries a gene (*muc*<sup>+</sup>), which in some strains (*recA*<sup>+</sup>/*lexA*<sup>+</sup>) have proven to participate in "SOS" DNA-repair. This repair pathway is induced by DNA damage and confers resistance to the lethal effects of many mutagens at the expense of increased mutability. Bacteria carrying pKM101 have therefore a higher spontaneous mutation rate.

#### *Escherichia coli*

Two strains were used:

*Escherichia coli* WP2*uvrA*

*Escherichia coli* WP2*uvrA*pKM101

These strains contain an ochre mutation in the *trpE* locus and can be mutated to tryptophan independence either by a base-pair reversion of an A-T base-pair in the *trpE* locus, or more likely, by a base-pair substitution within a number of transfer RNA loci elsewhere in the chromosome. The latter causes the original defect to be suppressed (ochre suppression) and involves only base-pair substitution transitions at G-C base-pairs.

Like the *uvrB* mutation in the *Salmonella* strains, the *uvrA* mutation causes the bacteria to be deficient in the excision of bulky lesions from the DNA, so, it is more readily mutated by certain agents (ultraviolet radiation, polycyclic hydrocarbons).

Additionally the WP2*uvrA*pKM101 strain contains the pKM101 plasmid, which confers resistance to ampicillin as described for the *Salmonella* strains TA98 and TA100.

#### 4.6. Bacterial cultures

The test strains of *Salmonella typhimurium* LT2 were obtained from Prof. B.N. Ames, Biochemistry Department, University of California, Berkeley, CA 94720, U.S.A.

*Escherichia coli* WP2*uvrA* was obtained from The National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland.

Both strains of *Escherichia coli* WP2 come from The National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland. WP2*uvrA* was obtained directly from this culture collection and WP2*uvrA*pKM101 through Covance Laboratories Ltd, Harrogate, North Yorkshire, England.

New batches of culture stocks frozen in 8% dimethyl sulphoxide are prepared at intervals from a central stock held in liquid nitrogen. They are regularly checked for appropriate amino acid requirement, spontaneous reversion rate, genetic characters and response to diagnostic mutagens.

Samples of each strain were grown up overnight in Nutrient broth in a 37 ± 1°C water bath with shaking. Fresh cultures were prepared before each test.

#### 4.7. S9 mix

Composition of a 10% V/V S9 mix (final concentrations):

Co-factors:

-phosphate buffer (0.2M, pH 7.4) .....	100 mM
-salts (1.65M KCl, 0.4 M MgCl).....	33 and 8 mM
-glucose-6-phosphate, mono-Na salt (0.2M) .....	5 mM
-NADP, di-Na salt (0.1M) .....	4 mM
S9 preparation .....	10% V/V

A freshly prepared solution of the co-factors was filter-sterilised by passage through a 0.2 µm membrane filter and mixed 9:1 (v/v) with freshly thawed still cold S9 preparation. This S9 mix was prepared freshly each day, and immediately used. Unused reagent was discarded.

#### 4.8. Test material

Serial dilutions of the sterile standard solution (4.1.) were prepared in sterile deionised water corresponding to the final dose levels:

5000 µg - 2500 µg - 1250 µg - 625 µg - 313 µg - 156 µg substance per ml.

The dilutions were prepared freshly each day just before use.

This range of doses was applied in experiments with respectively without S9.

#### 4.9. Top agar

0.6% soft agar was sterilised by autoclaving.

Bottles with 100 ml melted soft agar were kept at about 55°C and added 10 ml L-histidine/biotin solution for strains of *Salmonella* or 10 ml tryptophan solution for *Escherichia coli*. This molten agar was divided into 2 ml aliquots in sterile glass tubes and placed in a "Digital heatblock" (VWR) at 45 ± 1°C.

### 5. METHODS

#### 5.1. Treat and plate assay

This procedure was applied in test series with all strains of *Salmonella typhimurium*.

For each assay sterile tubes were added:

- 4 ml Nutrient broth
- 4 ml S9 mix or 0.2M phosphate buffer (pH 7.4)
- 1 ml bacterial culture
- 1 ml test substance solution (6 doses) or diagnostic mutagen solution (positive control) or sterile deionised water (solvent control).

These incubation mixtures were incubated with shaking at 37 ± 1°C for 3 hours.

After incubation all bacterial suspensions were washed 2 times by centrifugation for 10 minutes at 2500 rpm. After the first washing the bacterial pellets were resuspended in 5 ml phosphate buffer (pH 7.4, 0.2M) and finally they were re-suspended in 1 ml phosphate buffer.

Tubes with top agar were added 0.1 ml of all washed bacterial suspensions.

#### 5.2. Re-incubation

This procedure was applied in the third experiment with the strains *Salmonella typhimurium* TA98 and TA100 and *E.coli*WP2uvrApKM101.

Following the 3 hours exposure in the treat and plate procedure 0.1 mL of all incubation mixtures was added to tubes containing 10 mL nutrient broth. These incubation mixtures were incubated overnight with shaking at 37°C.

After incubation the cultures were centrifugated for 10 minutes at 2500 rpm and the bacterial pellets were resuspended in 2 ml phosphate buffer (pH 7.4, 0.2M). Tubes with top agar were added 0.1 ml of all cultures

#### 5.3. Plate incorporation assay

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This procedure was applied in the test series with *Escherichia coli* WP2uvrA.

Tubes with molten top agar were added:

100 µl of the dosing solutions of the test article or solvent (negative control) or standard mutagen (positive control).

100 µl bacterial culture.

500 µl S9 mix in test series with metabolic activation or 500 µl 0,2 M phosphate buffer in series without metabolic activation.

#### 5.4. Selective incubation

For each dose of the test substance and the standard mutagens 3 similar tubes with top agar were prepared and 5 tubes were prepared for the solvent control.

These tubes were poured on to minimal glucose agar plates. When the soft agar set, the plates were inverted and incubated at  $37 \pm 1^\circ\text{C}$  for about 48-72 hours. After incubation the numbers of revertant colonies were counted automatically ("Cardinal" - Perceptive Instruments). Plates with less than about 20 colonies were counted manually.

#### 5.5. Viable cell count

0.1 ml aliquots of a  $10^{-6}$  dilution of each bacterial suspension were poured on to minimal glucose agar plates (added the required amino acids in excess) in duplicates.

#### 5.6. Controls

The following controls were run with each experiment :

**Genotype** checking:

Sensitivity for crystal violet (rfa-character)- (except *E. coli*)

Sensitivity for Mitomycin C (uvrB).

Resistance to ampicillin (pKM101).

0.1 ml bacterial culture was spread on to complete agar medium. To the surface of the dried plate was added a disc of ampicillin/(Rosco Neo-Sensitabs) and two 6 mm  $\phi$  sterile filter discs, one with 10µl 0.1% crystal violet and the other with 10µl 0.01% Mitomycin C. The plate was incubated for 48-72 hours at  $37 \pm 1^\circ\text{C}$ .

#### Sterility of S9 mix:

0.1 ml S9 mix was plated on to complete medium and incubated for 48-72 hours at  $37 \pm 1^\circ\text{C}$ .

Diagnostic mutagens were used for each strain with and without S9 mix, as follows:

Mutagen	S9	Strain	µg/plate	µg/ml
MNNG	-	TA 1535		1.0
MNNG	-	TA 100		1.0
2-NF	-	TA 98		20.0
9-AA	-	TA 1537		2.0
ENNG	-	WP2uvrA	2.0	
ENNG	-	WP2uvrApKM101		5.0
2-AA	+	TA 98		5.0
2-AA	+	TA 1537		5.0
2-AA	+	TA 1535		5.0
2-AA	+	TA 100		5.0
2-AA	+	WP2uvrA	20.0	
2-AA	+	WP2uvrApKM101		20.0

## 6. RESULTS AND DISCUSSION

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### 6.1 Genetic characters

All strains used in these experiments were sensitive to Mitomycin C. All *Salmonella* strains were sensitive to crystal violet. Further the *Salmonella* strains TA98 and TA100 and *E.coli*WP2 uvrApKM101 were resistant to ampicillin. These results are as expected.

### 6.2 Diagnostic mutagens

In general the increases in revertant colony numbers induced by positive control chemicals were within the ranges experienced in our laboratory under the specific conditions applied in the present investigation (Appendix 2) and thereby confirming the sensitivity of the test system. In the first experiment with *S.typhimurium* TA 98 and third experiment with TA100 5 µg/ml 2-Aminoanthracene induced significant positive responses in the presence of S9, about 50 times the solvent control but in the low end of our historical data range. It is our experience that these responses to diagnostic mutagens are rather variable in the treat and plate assay.

### 6.3 Negative control levels

All negative control values presented in this report are within the normal ranges experienced in our laboratory with these strains of *Salmonella typhimurium* and *Escherichia coli* (Appendix 1). In general the spontaneous revertant levels are lower in a treat and plate assay than in the standard plate incorporation assay.

### 6.4 Asparaginase (Batch Number: PPV 24743)

The results are represented in Table 1-17

All investigations with the histidine requiring *Salmonella* strains were conducted applying a liquid culture method also called "treat and plate" assay (Table 1-12).

In the initial experiment the *Escherichia coli* strain WP2uvrA was treated with Asparaginase in the standard plate incorporation assay (Table 13). It was demonstrated that the Asparaginase test article significantly support growth of the tryptophan requiring *E.coli* strain by direct plate incorporation. The bacterial background lawn becomes increasingly dense and conspicuous with increasing doses of Asparaginase due to content of tryptophan. As a result, significant dose-related increases in the number of spontaneous revertants are observed in these two test series.

As a consequence, two new and independent experiments were conducted with the strain *E.coli* WP2uvrApKM101 applying the treat and plate assay (Table 14 and 15). This plasmid pKM101 containing strain was preferred since it is our experience that this strain is more sensitive to pro-mutagens in the presence of metabolic activation than the strain without this plasmid when exposed in liquid culture.

Asparaginase is a fluid enzyme preparation. Besides amino acids it contains an abundance of various nutrients, and composes a rich growth medium to the test bacteria. It is our experience, that in a treat and plate assay, where bacteria are exposed to different doses of such a test substance in separate liquid cultures for a certain time, the spontaneous revertant levels fluctuate more than in the direct "plate incorporation assay." Variation in the viable counts due to growth stimulation/inhibition in the treat and plate assay may cause some variation in the number of spontaneous revertant colonies.

These circumstances are reflected in the present study. No toxicity of the test substance to the bacteria is observed. On the contrary weak growth stimulation is evident to some extent in some of the test series demonstrated as increases in the viable count of exposed cultures compared to the solvent control. Concomitantly low numerical increases in the number of spontaneous revertant colonies are present as expected. They are all below a doubling and only exceeding 50% in just a few cases compared to the solvent control. These increases are hardly reproducible and are obviously not of biological importance.

In an attempt to demonstrate this relation between the viable count and revertant colony count an additional third experiment was carried out with the two *Salmonella* strains TA98

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and TA100 and *E. coli* WP2uvrApKM101 including a re-growth experiment of all cultures exposed in the treat and plate assay as described in section 5.2. No increases worth consideration were observed in any of these test series either before or after re-incubation.

We consider a test substance as positive in this treat and plate assay when it has induced at least a doubling in the mean number of revertants per plate compared to the appropriate solvent control in one or more of the strains, in the presence or absence of S9, if this response is dose related (at least 3 doses) and reproducible. If a numerical increases below a doubling is observed and if these increases is dose related and reproducible and not accompanied by significant increases in the viable bacterial count the result is considered as equivocal and need further clarification.

No treatments of any of the *Salmonella* and *E.coli* strains with Asparaginase resulted in any increases in revertant numbers that meets these criteria for a positive or equivocal response.

## 7. CONCLUSION

The results of the bacterial mutagenicity tests described in this report give no indication of the presence of mutagenic components in this preparation of Asparaginase (Batch No. PPY 24900), when tested under the conditions employed in this study.

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Table 1.

1. Experiment

*Salmonella typhimurium*, strain TA98. Treat and plate assay

µg per ml incubation mixture	Asparaginase <u>Without</u> metabolic activation (+S9)				Asparaginase <u>With</u> metabolic activation (+S9)				
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean	
5000	25	19	135	119	36	25	287	283	
	15		102		20		278		
	17				20				
2500	21	18	129	129	19	21	307	318	
	11		129		22		328		
	22				21				
1250	14	20	97	103	18	16	327	330	
	20		109		17		333		
	25				14				
625	22	19	112	101	27	20	335	359	
	17		90		14		383		
	18				18				
313	17	20	155	121	23	20	216	227	
	17		86		13		238		
	25				23				
156	17	18	168	165	17	23	183	211	
	18		161		29		238		
	18				24				
Solvent control	28	27	108	112	23	18	469	453	
	25				115				15
	29								18
	23								17
2- Nitrofluorene 20 µg	754	763	134	133					
	753		132						
	783								
2-Aminoanthracene 5 µg					815	805	171	179	
			816		186				
			783						

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

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Table 2.

## 1. Experiment

*Salmonella typhimurium*, strain TA1537. Treat and plate assay

µg per ml incubation mixture	Asparaginase <u>Without</u> metabolic activation (+S9)				Asparaginase <u>With</u> metabolic activation (+S9)					
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>			
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean		
5000	9	14	133	107	13	12	82	63		
	11		80		11		44			
	21				13					
2500	9	16	101	94	11	11	83	83		
	24		87		10		83			
	16				11					
1250	24	17	107	114	7	9	80	80		
	11		120		13		80			
	16				8					
625	12	12	90	102	18	12	85	80		
	11		114		8		74			
	13				11					
313	10	12	155	141	14	15	74	49		
	15		126		19		23			
	11				13					
156	17	17	107	117	7	11	59	66		
	15		126		16		72			
	20				10					
Solvent control	14	14	87	90	11	9	56	70		
	12				92				11	83
	14								9	
	13								7	
	18								8	
9-Amino-acridine 2 µg	3076	3167	140	126						
	3286		112							
	3138									
2-Amino-anthracene 5 µg					80					
					110	96	63	67		
					97		71			

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

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Table 3.

## 1. Experiment

*Salmonella typhimurium*, strain TA100. Treat and plate assay

µg per ml incubation mixture	Asparaginase Without metabolic activation (-S9)				Asparaginase With metabolic activation (+S9)			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	104	114	170	170	123	121	212	200
	119		170		124		188	
	120				117			
2500	120	107	131	134	101	108	256	256
	99		137		123		255	
	101				101			
1250	93	103	103	98	107	106	233	223
	114		92		109		213	
	102				103			
625	85	86	132	131	134	114	147	166
	82		130		102		185	
	91				107			
313	72	74	97	100	133	115	188	174
	86		103		113		160	
	65				99			
156	108	103	107	100	142	130	164	169
	108		92		118		173	
	94				130			
Solvent control	76	77	103	97	99	100	163	147
	64		90		104		131	
	85				97			
	92				104			
	70				97			
MNNG 1 µg	4600	4516	81	71				
	4276		61					
	4672							
2-AA 5 µg					856	886	107	98
					898		89	
					904			

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

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Table 4.

## 1. Experiment

*Salmonella typhimurium*, strain TA1535. Treat and plate assay

µg per ml incubation mixture	Asparaginase Without metabolic activation (±S9)				Asparaginase With metabolic activation (+S9)			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	11	9	176 201	189	3	5	265 260	263
	7				6			
	8				5			
2500	4	5	144 141	143	6	6	(81) (75)	*) (78)
	7				6			
	5				5			
1250	6	6	197 206	202	7	6	322 301	312
	6				3			
	6				7			
625	6	6	124 142	133	1	2	248 231	240
	3				4			
	9				2			
313	6	4	115 108	112	6	4	240 229	235
	4				2			
	3				5			
156	6	5	159 161	160	3	4	313 304	309
	5				4			
	5				4			
Solvent control	7	7	140 128	134	6	4	264 250	257
	8				4			
	9				1			
	6				4			
	4				5			
MNNG 1 µg	5823	5508	126 128	127	152	141	130 136	133
	5256				157			
	5446				114			
2-Amino-anthracene 5µg								

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

\*) Determination apparently vitiated by error. The bacterial background lawn on revertant plates did not reveal a reduction in viable cells compared to solvent control

Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

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Table 5.

## 2. Experiment

*Salmonella typhimurium*, strain TA98. Treat and plate assay

µg per ml incubation mixture	Asparaginase <u>Without</u> metabolic activation (+S9)				Asparaginase <u>With</u> metabolic activation (+S9)			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	27	23	15	10	37	33	236	229
	23		5		30		221	
	20				31			
2500	31	23	11	12	38	37	239	247
	21		13		36		254	
	18				37			
1250	24	21	3	2	26	24	248	232
	21		0		21		216	
	19				25			
625	21	21	28	21	24	21	184	191
	25		14		23		197	
	18				16			
313	25	21	11	7	19	16	209	191
	19		3		12		173	
	20				17			
156	18	22	5	13	19	21	223	223
	23		20		24		222	
	26				20			
Solvent control	28	20	5	14	21	21	200	189
	18		18		22		177	
	23				20			
	13				25			
	17				18			
2-Nitro-fluorene 20 µg	903	851	16	12				
	808		7					
	843							
2-Amino-anthracene 5 µg					1289	1338	148	144
					1312		139	
					1413			

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

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Table 6.

## 2. Experiment

### *Salmonella typhimurium*, strain TA1537. Treat and plate assay

µg per ml incubation mixture	Asparaginase <u>Without</u> metabolic activation (±S9)				Asparaginase <u>With</u> metabolic activation (+S9)			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	19	15	163	174	19	18	145	153
	12		185		20		158	
	15				16			
2500	9	12	153	153	12	17	113	119
	11		152		16		125	
	15				23			
1250	11	13	159	166	22	18	125	135
	17		172		18		144	
	12				15			
625	13	11	118	119	18	16	99	78
	9		119		13		56	
	10				16			
313	9	9	161	164	16	15	93	100
	10		167		15		107	
	7				14			
156	8	14	184	183	19	14	101	103
	21		182		9		104	
	12				14			
Solvent control	7	10	134	127	21	16	102	108
	10		119		16		113	
	8				17			
	10				10			
9-Amino-acridine 2 µg	1266	1386	92	103				
	1395		114					
	1496							
2-Amino-anthracene 5 µg					193	179	75	65
					180		55	
					164			

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

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Table 7.

2. Experiment

*Salmonella typhimurium*, strain TA100. Treat and plate assay

µg per ml incubation mixture	Asparaginase Without metabolic activation (±S9)				Asparaginase With metabolic activation (+S9)			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	106	89	70	63	261	236	90	79
	80		55		223		67	
	82				223			
2500	146	144	153	136	201	236	190	182
	151		118		245		173	
	136				261			
1250	150	123	155	139	255	228	161	144
	117		123		206		126	
	102				224			
625	97	96	97	97	194	186	99	107
	92		96		205		115	
	99				159			
313	99	107	89	87	215	211	85	76
	118		85		206		67	
	104				213			
156	117	122	128	128	209	197	128	116
	118		128		202		104	
	131				180			
Solvent control	102	98	115	115	136	142	76	65
	129		115		175		53	
	92				121			
	77				131			
MNNG 1 µg	2345	2464	89	76				
	2559		62					
	2489							
2-AA 5 µg					625	622	34	41
			633		48			
			607					

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

Table 8.

2. Experiment

*Salmonella typhimurium*, strain TA1535. Treat and plate assay

µg per ml incubation mixture	Asparaginase Without metabolic activation (-S9)				Asparaginase With metabolic activation (+S9)			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	5	4	53	54	3	4	169	165
	4		54		2		161	
	2				8			
2500	4	4	113	129	10	7	271	268
	4		144		6		264	
	4				6			
1250	4	3	122	128	7	6	340	311
	2		134		3		281	
	3				8			
625	4	3	134	129	9	6	250	236
	4		124		5		222	
	2				5			
313	4	5	123	121	7	5	195	176
	4		118		4		156	
	6				3			
156	4	6	103	109	3	4	181	183
	7		114		5		184	
	6				4			
Solvent control	4	3	106	119	5	5	169	173
	3		132		4		177	
	2				5			
	2				6			
	4				7			
MMNG 1.0 µg	4069	4145	93	98				
	4148		102					
	4219							
2-Amino- anthracene 5µg					88	77	86	94
			88		102			
			55					

1) Number of revertant colonies per plate  
 2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.  
 Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

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Table 9.

3. Experiment

*Salmonella typhimurium*, strain TA98. Treat and plate assay with re-incubation

µg per ml incubation mixture	Asparaginase							
	Without metabolic activation (+S9)							
	Treat and plate				Re-incubation			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean	
5000	10		255		10		60	
	17	17	211	233	6	9	71	66
	24				10			
2500	11		204		14		58	
	19	16	184	194	13	16	65	62
	17				20			
1250	20		202		18		49	
	13	15	201	202	11	16	71	60
	13				18			
625	11		154		14		75	
	16	15	164	159	8	11	90	83
	18				10			
313	13		177		15		87	
	13	13	157	167	15	13	85	86
	12				8			
156	9		189		21		112	
	12	14	215	202	17	19	118	115
	22				18			
Solvent control	5				16			
	16		177		15		67	
	19	14	142	160	24	16	93	80
	13				22			
	18				16			
2-Nitro-fluorene 20 µg	682		141		170		59	
	694	690	147	144	170	176	39	49
	695				189			

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

\* An error must have occurred in the procedure of spreading the viable cells to plates.

Table 10

3. Experiment

*Salmonella typhimurium*, strain TA98. Treat and plate assay with re-incubation

µg per ml incubation mixture	Asparaginase With metabolic activation (+S9)							
	Treat and plate				Re-incubation			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	32	23	97	75	-	- *)	132	118
	18		52		-		104	
	18				-			
2500	32	30	131	142	-	- *)	93	100
	29		153		-		107	
	29				-			
1250	23	26	136	136	20	20	55	65
	34		- *)		12		74	
	21				28			
625	22	22	134	142	28	22	74	82
	20		150		21		90	
	23				16			
313	18	23	182	143	22	29	83	80
	32		104		33		76	
	20				32			
156	25	25	168	174	33	29	74	70
	25		180		26		66	
	26				29			
Solvent control	27	22		154	21	22		*)
	20		150		20		-	
	25		158		22		-	
	18				19			
	22				26			
2-Amino-anthracene 5 µg	1165	1153	83	81	236	234		*)
	1070		79		233		-	
	1224				232		-	

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

\*) No determination due to plating error.

000086

Table 11.

3. Experiment

*Salmonella typhimurium*, strain TA100. Treat and plate assay with re-incubation

µg per ml incubation mixture	Asparaginase							
	Without metabolic activation (±S9)							
	Treat and plate				Re-incubation			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	139		77		119		60	
	142	133	52	65	64	86	72	66
	118				75			
2500	101		94		117		60	
	120	118	85	90	98	97	60	60
	134				77			
1250	113		81		130		79	
	112	116	81	81	114	121	79	79
	123				119			
625	115		94		52		64	
	109	116	85	90	75	68	80	72
	123				76			
313	98		82		96		60	
	85	95	84	83	85	93	35	48
	102				99			
156	137		107		91		70	
	110	121	104	106	126	104	80	75
	115				94			
Solvent control	137				85			
	*)		64		76		74	
	*)	137	93	79	99	84	55	65
	*)				90			
MNNG 1 µg	3497		47		1239		44	
	3700	3599	54	51	1325	1293	41	43
	*)				1315			

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

\*) Plating error.

Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

Table 12

3. Experiment

*Salmonella typhimurium*, strain TA100. Treat and plate assay with re-incubation

µg per ml incubation mixture	Asparaginase With metabolic activation (+S9)							
	Treat and plate				Re-incubation			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	85	85	115	107	65	58	79	84
	97		98		48		89	
	74				60			
2500	82	90	126	126	60	64	85	76
	97		125		66		66	
	90				65			
1250	113	104	150	151	48	53	119	101
	98		152		58		82	
	102				52			
625	98	94	164	164	54	50	107	103
	102		164		53		98	
	82				43			
313	87	85	157	162	54	50	131	120
	- *)		167		49		109	
	83				48			
156	- *)	96	180	170	71	58	48	63
	- *)		160		54		77	
	96				48			
Solvent control	75	74		120	47	55		75
	83		117		64		71	
	85		123		66		79	
	69				33			
	58				63			
2-Amino-anthracene 5 µg	459	458	108	120	166	200	77	69
	487		131		207		60	
	428				227			

1) Number of revertant colonies per plate  
 2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.  
 \*) Plating error.

000088

Table 13.

1. Experiment. Plate incorporation assay

*Escherichia coli*, strain WP2uvrA

Test substance concentration (µg/plate)	Asparaginase_		Asparaginase	
	Without metabolic activation (+S9)		With metabolic activation (+S9)	
	Revertants <sup>1)</sup>		Revertants <sup>1)</sup>	
	Single plates	Mean	Single plates	Mean
5000	113	104	137	120
	96		131	
	104		92	
2500	69	90	164	167
	99		164	
	101		174	
1250	59	79	159	140
	87		132	
	92		129	
625	60	69	85	71
	76		61	
	70		66	
313	71	66	103	109
	67		103	
	60		121	
156	74	69	102	106
	73		117	
	59		99	
Solvent control	54	50	83	76
	52		99	
	50		77	
	45		71	
	51		49	
ENNG 2 µg	234	238		
	222			
	257			
2-Amino-anthracene 20 µg			138	347
			332	
			571	
Viable count of bacterial cultures x 10 <sup>7</sup>	224	230	224	230
	236		236	

1) Number of revertant colonies per plate

\*) Dose related increase in the density of the background lawn of auxotroph bacteria

\*\*\*) The determination subjected to uncertainty due to increased density of the bacterial background lawn.

Abbreviation: ENNG = 1-Ethyl-3-Nitro-1-Nitrosoguanidine.

Table 14.

1. Experiment. Treat and plate assay  
*Escherichia coli*, strain WP2uvrApKM101

µg per ml incubation mixture	Asparaginase Without metabolic activation (±S9)				Asparaginase With metabolic activation (+S9)					
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>			
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean		
5000	399	412	410	369	332	342	422	420		
	407		328		354		418			
	429				340					
2500	275	290	303	283	357	333	303	318		
	308		263		314		332			
	287				329					
1250	261	261	156	195	294	330	374	380		
	266		233		332		385			
	256				364					
625	248	263	240	228	326	317	396	371		
	274		215		304		346			
	266				321					
313	274	273	194	180	302	318	312	326		
	270		166		317		340			
	275				335					
156	260	264	238	216	294	299	348	352		
	245		193		289		355			
	287				315					
Solvent control	271	253	210	200	283	274	309	328		
	219				189				290	347
	242								256	
	275								276	
ENNG 5.0 µg	1525	1555	145	154						
	1579		162							
	1560									
2-Amino-anthracene 20.0 µg					1747	1681	240	247		
			1684		254					
			1611							

1) Number of revertant colonies per plate  
 2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.  
 Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

000090

Table 15.

2. Experiment. Treat and plate assay  
*Escherichia coli*, strain WP2uvrApKM101

µg per ml incubation mixture	Asparaginase Without metabolic activation (+S9)				Asparaginase With metabolic activation (+S9)			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	335	313	316	323	277	289	337	328
	292		329		304		318	
	312				286			
2500	248	249	217	222	252	252	438	430
	253		227		260		421	
	245				243			
1250	267	279	237	237	302	302	260	253
	283		237		325		245	
	287				280			
625	244	248	206	195	348	386	245	218
	242		184		402		190	
	258				408			
313	244	250	221	236	286	294	184	194
	249		250		291		204	
	256				305			
156	261	260	244	225	308	301	326	292
	247		206		319		257	
	272				277			
Solvent control	244	236		183	238	259		260
	228		202		258		238	
	243		163		267		282	
	218				286			
	247				245			
ENNG 5.0 µg	2140	2207	191	187				
	2284		183					
	2196							
2-Amino- anthracene 20.0µg					1066	1110	220	230
			1136		240			
			1127					

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

Abbreviation: MNNG = N-Methyl-N'-Nitro-Nitrosoguanidine.

Table 16.

3. Experiment. Treat and plate assay with re-incubation  
*Escherichia coli*, strain WP2uvrApKM101.

µg per ml incubation mixture	Asparaginase Without metabolic activation (+S9)							
	Treat and plate				Re-incubation			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	217		211		130		200	
	216	225	173	192	136	126	190	195
	242				112			
2500	206		151		157		207	
	209	207	145	148	146	149	190	199
	206				145			
1250	193		130		120		163	
	201	188	157	144	126	125	145	154
	171				128			
625	202		123		142		139	
	209	206	141	132	126	129	114	127
	207				120			
313	166		136		129		153	
	205	194	108	122	131	129	124	139
	212				126			
156	185		112		124		164	
	195	194	109	111	67	107	148	156
	201				131			
Solvent control	171				118			
	157		92		107		212	
	186	169	93	93	136	119	189	201
	155				113			
ENNG 5.0 µg	177				120			
	1278		98		1014		157	
	1321	1264	126	112	1018	988	132	145
	1192				931			

1) Number of revertant colonies per plate  
 2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.  
 Abbreviation: ENNG = N-Ethyl-N'-Nitro-Nitrosoguanidine.

000092

Table 17

3. Experiment. Treat and plate assay with re-incubation

*Escherichia coli*, strain WP2uvrApKM101

µg per ml incubation mixture	Asparaginase With metabolic activation (+S9)							
	Treat and plate				Re-incubation			
	Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>		Revertants <sup>1)</sup>		Viable cells <sup>2)</sup>	
	Single plates	Mean	Single plates	Mean	Single plates	Mean	Single plates	Mean
5000	260		315		147		244	
	242	260	324	320	130	137	232	238
	277				134			
2500	259		213		120		206	
	258	260	202	208	141	128	195	201
	263				122			
1250	265		178		121		183	
	260	260	167	173	135	125	202	193
	255				120			
625	223		119		125		190	
	251	239	155	137	125	127	158	174
	244				130			
313	178		166		140		188	
	229	201	155	161	115	123	251	220
	195				113			
156	204		131		109		175	
	216	210	150	141	120	119	193	184
	211				129			
Solvent control	223				81			
	204		191		114		212	
	232	208	191	191	92	104	210	211
	202				117			
2-Amino-anthracene 20 µg	179				117			
	945		125		287		169	
	925	917	115	120	270	274	169	169
	881				266			

1) Number of revertant colonies per plate

2) Corresponding to no. of viable cells x10<sup>6</sup> on revertant plates.

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## APPENDIX 1

### Historical control data

**Negative control** (purified water) ranges for *S. typhimurium* strains and *E.coli* WP2uvrApKM101 obtained in the treat and plate assay (SOP: TOX-SM-0809-10) and ranges obtained with *E.coli* WP2uvrA in the plate incorporation assay (SOP: TOX-SM-0806).

Strain	S9	Number of *) determinations	Mean number of revertants per plate	SD	Range **)	
					lower	upper
TA1535	÷	20	6	± 1.8	3	9
	+	20	6	± 2.0	2	9
TA100	÷	23	75	± 18	38	137
	+	15	100	± 32	44	174
TA1537	÷	17	11	± 5	4	22
	+	23	12	± 5	5	25
TA98	÷	25	17	± 5	7	27
	+	26	21	± 6	12	41
WP2 uvrA	÷	23	41	± 11	17	60
	+	23	47	± 15	20	84
WP2uvrA pKM101***	÷	12	197	± 37	135	253
	+	12	246	± 58	141	339

\*) The above are pooled data from a number of independent determinations selected from studies conducted within a year. Only determinations, which were obviously vitiated by errors, have been omitted.

\*\*) Ranges stated are the maximum and minimum mean spontaneous revertant counts from the data sets sampled

\*\*\*) The data are pooled from a number of independent determinations selected from studies conducted within 2004 to 2006. Only determinations, which were obviously vitiated by errors, have been omitted

Historical control data

Positive control ranges for *S. typhimurium* strains and *E.coli* WP2uvrApKM101 obtained in the treat and plate assay (SOP: TOX-SM-0809-10) and ranges obtained with *E.coli* WP2uvrA in the plate incorporation assay (SOP: TOX-SM-0806).

Strain	S9	Number of *) determinations	Chemical	Mean number of revertants per plate	SD	Range **)	
						lower	upper
TA1535	÷	20	MNNG 1 µg/mL	3633	1926	562	5783
	+	18	2-AA 5 µg/mL	126	33	53	197
TA100	÷	20	MNNG 1 µg/mL	2893.2	1580	493	4846
	+	12	2-AA 5 µg/mL	1125	564	205	2312
TA1537	÷	20	9-AA 2 µg/mL	827	529	96	1809
	+	25	2-AA 5 µg/mL	145	55	25	228
TA98	÷	23	2-NF 20 µg/mL	851	149	609	1264
	+	25	2-AA 5 µg/mL	1846	589	203	2682
WP2 uvrA	÷	24	ENNG 2 µg/plate	675	403	110	1635
	+	24	2-AA 20 µg/plate	564	223	228	1051
WP2uvrA pKM101***	÷	9	ENNG 5 µg/mL	1542	361	1210	2207
	+	12	2-AA 20 µg/mL	1253	247	917	1681

Abbreviations:

- 2NF: 2-nitrofluorene
- MNNG: N-methyl-N'-nitro-N-nitrosoguanidine
- ENNG: N-Ethyl-N'-nitro-N-nitrosoguanidine
- 2-AA: 2-aminoanthracene
- BaP: Benzo(a)pyrene
- 9-AA: 2-aminoacridine

\*) The above are pooled data from a number of independent determinations from studies conducted within 2005 and 2006. Only determinations, which were obviously vitiated by errors, have been omitted.

\*\*) Ranges stated are the maximum and minimum mean induced revertant colony counts from the data sets sampled

\*\*\*) The data are pooled from a number of independent determinations selected from studies conducted within 2004 to 2006. Only determinations, which were obviously vitiated by errors, have been omitted.

### APPENDIX 3

#### PREPARATION OF MEDIA

1. **Top-agar - histidine-deficient soft agar**

Agar, Merck	0.6 g
NaCl	0.6 g
Distilled water to	100 ml

The medium was autoclaved for 15 minutes at 121°C. After cooling to about 60°C, 10 ml of a sterile aqueous solution of 0.5 mM biotin - 0.5 mM histidine was added aseptically.

2. **Nutrient broth - histidine-rich broth**

Difco nutrient broth	8 g
NaCl	5 g
Distilled water to	1 litre

The medium was autoclaved for 15 minutes at 121°C.

3. **Nutrient agar - histidine-rich agar medium**

Agar, Merck	15 g
Oxoid nutrient broth No. 2	25 g
Distilled water to	1 litre

The medium was autoclaved for 15 minutes at 121°C.

4. **Minimal medium**

This was Vogel-Bonner minimal "E" medium with 2% glucose, prepared as follows :

Solution A (Vogel-Bonner medium E, 20X)

MgSO <sub>4</sub> 7H <sub>2</sub> O	4 g
Citric acid, monohydrate	40 g
K <sub>2</sub> HPO <sub>4</sub>	200 g
NaH <sub>2</sub> NH <sub>4</sub> 4H <sub>2</sub> O	70 g
Distilled water to	1000 ml

The solution was sterilized by filtration.

Solution B (40% glucose)

Glucose	40 g
Distilled water to	100 ml

This solution was sterilized by filtration.

Solution C (Agar base)

Agar, Merck	16.7 g
Distilled water to	1000 ml

Solution C was autoclaved for 15 minutes at 121°C. After cooling to 60°C, 450 ml of solution C was aseptically added 25 ml solution A and 25 ml solution B.

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Appendix 5

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**AN EVALUATION OF THE BUDGET METHOD  
FOR SCREENING  
FOOD ADDITIVE INTAKE**

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**Summary Report**

Prepared under the responsibility  
of ILSI Europe Food Chemical  
Intake Task Force

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***AN EVALUATION OF THE BUDGET METHOD  
FOR SCREENING FOOD ADDITIVE INTAKE***

SUMMARY REPORT PREPARED UNDER THE RESPONSIBILITY OF  
ILSI EUROPE FOOD CHEMICAL INTAKE TASK FORCE

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APRIL 1997

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An adaptation of the Budget Method developed by Søren Hansen (1966) of the National Food Agency of Denmark has been suggested as an appropriate screening method for determining priorities for monitoring the consumption and use of additives as required under European Union (EU) Directives 94/35/EC, 94/36/EC and 95/2/EC. Before it is accepted in the EU for such a purpose, however, the underlying assumptions should be examined to evaluate the potential for error in the use of results. Clearly, the Budget Method must minimise Type II (false negative) errors which could result in unchecked use of a substance that should have been a priority for monitoring. At the same time, Type I (false positive) errors should be as low as possible to prevent unnecessary expenditure of time and resources in pursuit of more detailed intake estimates.

This report summarises work performed by TAS (Technical Assessment Systems) International at the request of the International Life Sciences Institute - ILSI Europe Food Chemical Intake Task Force. The aim of the report was to evaluate the validity of assumptions on which the Budget Method is based and to assess the potential for Type I and II errors in using the method to establish additive monitoring priorities. The report should be read in conjunction with the references cited herein and listed at the end of this report.

**Figure 2. Assumptions made in screening additive intake using the Budget Method: Additives used in beverages.**

Maximum intake of liquids (other than milk) is 100 ml/kg body weight/day.  100 ml = 1/10 litre	
<p><i>The Theoretical Maximum Daily Intake (TMDI) of an additive can be calculated as:</i></p> <p style="text-align: center;"> <math display="block">\frac{\text{Maximum use level (mg additive/1 beverage)}}{10}</math> </p> <p style="text-align: center;"> <math display="block">\frac{\text{Maximum use level (mg additive/1 beverage)}}{40}</math> </p>	<p style="text-align: center;"><i>If:</i></p> <p>The additive is used in all non-milk beverages and the maximum amount of non-milk beverages consumed is:</p> <p style="text-align: center;"> <math display="block">\frac{1/10 \text{ litre}}{\text{kg body weight}}</math> </p> <p>The additive is used only in soft drinks and maximum soft drink intake is 25% of non-milk beverage intake.</p>

## 2. BUDGET METHOD

The Budget Method was designed to compare food additive ADIs with "ceilings of use" calculated on the basis of maximum consumption of food and beverages potentially containing the additives (Hansen 1966, 1979). In budget calculations for additives used in both solid foods and beverages, the ADIs are split into two fractions. The proportion of the ADI allocated to food and the proportion allocated to beverages are decided arbitrarily to accommodate technological requirements.

The proposal for use of the Budget Method calls for calculation of TMDIs using the assumptions outlined in Figures 1 and 2. Figure 1 describes the factors used and the assumptions made in estimating intake of additives from solid foods. Figure 2 describes the factors used and the assumptions made when estimating intake of additives from beverages.

## 3. METHODS

TAS International examined data from nationwide food consumption surveys conducted in the United Kingdom, former West Germany, and the United States (Figure 3). It examined whether Budget Method assumptions regarding energy intake, energy density of food and beverage soft drink consumption provide a valid basis for screening additive intake. Because the Budget Method assumes that ADIs for additives relate to average intake over a lifetime, the validity of assumptions was assessed on the basis of population averages rather than on high-level intake.

Because of limitations posed by the availability of European survey data, Budget Method assumptions regarding proportions of the diet accounted for by processed foods potentially containing additives could not be tested. The UK surveys were conducted on specific age groups, and results therefore were summarised on the basis of the UK age groups and on broad food groups.

## 4. CASE STUDIES

To evaluate the potential for Type I and II errors in using the Budget Method, case studies were conducted for two hypothetical food additives with different characteristics and proposed uses (Tables 1 and 2).

**Table 1. Case Study of Additive 1: Used at similar levels in a broad range of foods consumed by a significant portion of the population.**

<i>Food Category</i>	<i>Use limit (mg/kg food)</i>
Breads	50
Baked Products	50
Pastas	30
Cereals	50
Rice	30

**Table 2. Case Study of Additive 2: Used in varying concentrations in a range of foods consumed by specific segments of the population.**

<i>Food Category</i>	<i>Use limit (mg/kg food or mg/l beverage)</i>
Soft Drinks	350
Biscuits	1000
Confectionery	500

Maximum permitted use levels in specific food groups were selected to reflect typical use levels.

Food Additive 1 was designed to be representative of an additive used at similar levels in a broad range of foods

**Figure 3. Food consumption survey data used in Budget Method validation.**

#### ***United Kingdom***

Data used in the analyses were taken from the following summary sources of survey data published by the Ministry of Agriculture, Fisheries and Food:

- *Food and Nutrient Intakes of British Infants Ages 6-12 Months*; 1986; 488 infants; based on 7-day food intake records.
- *National Diet and Nutrition Survey: Children Ages 1.5-4.5 years*; July 1992 and June 1993; 1,675 children; based on 4-day food intake records.
- *The Diets of British School Children*; 1983; 3,581 children ages 10-11 and 14-15; based on 7-day food intake records.
- *The Dietary and Nutritional Survey of British Adults*; October 1986 - August 1987; 2,197 adults ages 16-64; based on 7-day food intake records.

#### ***Former West Germany***

National Consumption Study (NVS); October 1985-January 1989. Seven-day weighed-intake data were collected from more than 25,000 individuals 4 years of age and older, using a system consisting of more than 6,000 food codes. Data used in the present analyses were taken from a dataset containing records of average daily intake of foods in 90 summary food groups by individual survey respondents.

#### ***United States***

US Department of Agriculture 1989-90, 1990-91 and 1991-92 Continuing Surveys of Food Intake by Individuals (CSFII). Together, the CSFII surveys measured dietary intake of more than 11,000 individuals over a 3-day period. Although these data clearly do not directly reflect European food intake patterns, it was believed that the extensive information on intake by individuals could be of potential value in examining basic Budget Method assumptions regarding food additive intake. For example, neither the UK surveys nor the German survey collected data on individuals in all age groups; the US data provide supplementary data on total population intake.

consumed by a significant proportion of the population. Food Additive 2 was designed to be representative of an additive used in varying concentrations in a range of foods consumed by specific segments of the population (e.g., an intense sweetener).

The Budget Method TMDI for Additive 2 was calculated allocating half of the budget to soft drinks and half to "high additive" solid foods (biscuits and confectionery), using a liquid intake factor of 40 and a food consumption factor of 160 (Figures 1 and 2).

Food consumption survey-based intake estimates for Additives 1 and 2 were calculated as precisely as possible given the limitations of the survey data. Additive use levels were applied to food consumption data in the categories shown in Tables 1 and 2.

Limitations on the availability of the survey data used in these analyses precluded calculation of per-user intake. However, it was assumed that all people will be consumers of the additives in question at some point in their lifetimes, and *per capita* estimates were therefore judged to be representative of lifetime intake. Because it is generally believed that the Budget Method generates conservative estimates of intake, TMDIs were compared with 95th percentile *per capita* intake. Where survey data did not permit assessment of 95th percentile *per capita* intake, the intake values were estimated using the Bernier method (Bernier et al. 1994) at three times the mean.

## 5. RESULTS

### 5.1 Energy intake

The survey results indicated that the Budget Method assumptions of 100 kcal/kg body weight energy intake for 1-year-olds and of 50 kcal/kg body weight for the general population overestimate actual lifetime average energy intake by a small margin.

### 5.2 Energy density of food

In developing the Budget Method, Hansen (1966) assumed that 50 g of food have an energy value equal to 100 kcal, which is equivalent to 2 kcal/g. In contrast, UK and US survey data indicate that the mix of food consumed, on average, is of slightly lower energy density (the German data did not permit calculation of energy density).

The Budget Method is therefore based on an overestimate of energy density of foods consumed by the general population. The extent of the overestimate would be even greater for subpopulations consuming large quantities of low-calorie foods

### 5.3 Beverage and soft drink consumption

Budget Method calculations for additive intake from beverages are based on the assumption that 100 ml/kg body weight of liquids, excluding milk, represents maximum consumption. The maximum soft drink consumption is assumed to be 25% of this consumption, or 25 ml/kg body weight/day.

Beverage consumption data are shown for selected age groups in Table 3. In most cases these are clearly overestimated by the Budget Method assumptions. It is likely that UK soft drink consumption by 1.5-4.5-year-old children is higher than that by children of comparable ages in Germany and the USA because water-diluted rose hip, blackcurrant and other fruit cordials popular with young children in the UK are included in summarised soft drink consumption data.

### 5.4 Comparison of TMDIs with Additive Intake Estimates Based on Food Consumption Survey Data

Budget Method TMDIs calculated for Food Additives 1 and 2 (0.63 and 15.00 mg/kg body weight) are above the 95th percentile *per capita* estimates of intake for all three national surveys investigated (Table 4), indicating that the budget method provides a conservative first estimate of intake.

It is unlikely that Type I or II errors would occur in the assignment of monitoring priority for additives like Food Additive 1 using the Budget Method. Additive 1 was intended to be representative of an additive used at similar levels in a broad range of foods consumed by a significant proportion of the population.

The scenario for additives like Food Additive 2 is different. This hypothetical additive was intended to be representative of an additive used in varying concentrations in a range of foods consumed by specific segments of the population (e.g., an intense sweetener). The Budget Method TMDI for Food Additive 2 is three to five times the survey-based estimates of intake, indicating a potential for Type I (false positive) errors. This means that the Budget Method is likely to falsely identify this type of additive as warranting further attention.

Table 3. Beverage and soft drink consumption estimates based on UK, West German and US survey data.

Age Group	Mean Per Capita Intake of Non-Milk Beverages and of Soft Drinks (g/kg body weight/day)					
	UK		West German		US	
	All non-milk beverages	Soft drinks only	All non-milk beverages	Soft drinks only	All non-milk beverages	Soft drinks only
Total population	NA <sup>1</sup>	NA	17 <sup>2</sup>	2 <sup>2</sup>	13	4
6 - 12 months	18	4	NA	NA	17	2
1.5-4.5 years	31	25 <sup>3</sup>	NA	NA	18 <sup>4</sup>	5 <sup>4</sup>
10 -11 years	8	4	16	5	11	5
14-15 years	9	3	13	4	11	6
16-64 years	23	2	18	2	13	4

(1) NA = not applicable (population group not included in survey).

(2) Ages 4+.

(3) Water-diluted rose hip, blackcurrant, and other fruit cordials popular with young children in the UK are included in summarised soft drink intake data.

(4) Ages 1-5 years.

Table 4. Budget Method TMDI and survey-based intake estimates for case study Additives 1 and 2.

Food Additive	Budget Method TMDI (mg/kg body weight)	Intake Estimates Based on Food Consumption Survey Data	
		Population	95th percentile per capita intake (mg/kg body weight/day)
1	0.63	UK <sup>1</sup>	0.34
		West German <sup>2</sup>	0.40
		US	0.43
2	15.00	UK	2.88
		West German	4.42
		US	5.12

(1) Ages 16-64; 95th percentile intake for this population computed as (mean intake x 3), as recommended by Bernier et al. (1994).

(2) Ages 4+.

## 6. CONCLUSIONS

The Budget Method assumptions regarding energy intake, energy density of foods, and beverage and soft drink consumption using UK, German and US national food consumption data were reviewed, and the following conclusions were reached:

- Assumptions regarding energy intake and beverage and soft drink consumption by the general population are overestimates of actual average levels. These probably add to the overall conservatism of the Budget Method.
- The Budget Method assumption regarding the energy density of foods may result in small overestimates. This probably detracts slightly from the overall conservatism of the method.

In the two case studies, Budget Method TMDIs were larger than survey-based *per capita* additive intake estimates, providing evidence that the Budget Method produces overestimates of additive intake. The potential for Type II (false negative) errors is therefore judged to be small whereas the potential for Type I (false positive) errors seems to be relatively large.

Thus, the Budget Method appears to be a satisfactory conservative first screen for establishing priorities for monitoring the intake of food additives, based on potential average lifetime food consumption.

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## 7. REFERENCES

Bernier JJ, Causeret J, Hoellinger H, Suschetet M. Méthode pour assurer, au sein d'une population, le non-dépassement de la dose journalière admissible des additifs alimentaires. *Med Nutri* 1994;1:9-17

Douglass JS, Barraj LM, Tennant DR, et al. Evaluation of the budget method for screening food additive intakes. *Food Addit Contam* 1997 (in press)

Hallas-Møller T. Using the budget method as a quick screening method for identifying food additives for which further monitoring is not warranted on health grounds. Draft report prepared for the SCF, 1 June 1995

Hansen SC. Acceptable daily intake of food additives and ceiling on levels of use. *Food Cosmet Toxicol* 1966;4:427-432

Hansen SC. Conditions for use of food additives based on a budget for an acceptable daily intake. *Food Protect* 1979;42:429-432

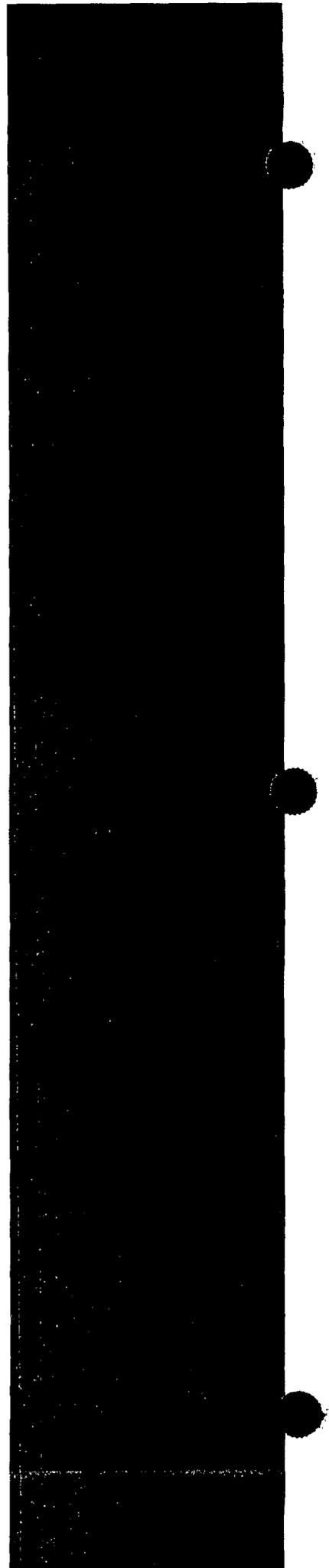


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**SUBMISSION END**

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## *Reference List for Industry Submission, GRN 000201*

<i>Pages</i>	<i>Author</i>	<i>Title</i>	<i>Publish Date</i>	<i>Publisher</i>	<i>BIB_Info</i>
000029 - 000042	Pariza, Michael W.; Johnson, Eric A.	Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century	April 12, 2001	Regulatory Toxicology and Pharmacology	Volume 33, pgs 173-186
000049 - 000052	Barbesgaard, Peder; Heldt-Hansen, Hans Peter; Diderichsen, Berge	On the safety of Aspergillus oryzae: a review	1992	Applied Microbiology and Biotechnology	Volume 36, pgs 569-572

*NA- Not applicable*

**Merker, Robert I**

AM



**From:** LOBG (Lori Gregg) [LOBG@novozymes.com]  
**Sent:** Thursday, July 27, 2006 9:12 AM  
**To:** Merker, Robert I  
**Subject:** RE: long-delayed thoughts: GRN 201  
**Attachments:** REG\_application ltr asp.pdf

Dear Bob,

Attached please find a letter that discusses the intended applications for the asparaginase and addresses the points in your #1 below.

As I mentioned by phone, my colleague in DK is on vacation until August 7. When he returns I should be able to provide a little more information on the identity of the enzyme.

If you have any questions, please don't hesitate to contact me.

Best Regards,

*Lori Gregg*  
Regulatory Specialist

**Novozymes North America, Inc.**  
77 Perry Chapel Church Road, Box 576  
Franklinton, NC 27525  
Phone: 919-494-3151  
Fax: 919-494-3420

-----Original Message-----

**From:** Merker, Robert I [mailto:robert.merker@fda.hhs.gov]  
**Sent:** Thursday, July 20, 2006 2:05 PM  
**To:** LOBG (Lori Gregg)  
**Subject:** long-delayed thoughts: GRN 201

Good afternoon, Ms. Gregg.

After some internal discussion, here's what we think would help facilitate the review of the notice on the asparaginase from *A. oryzae*.

1. That the uses in the cover letter and GRAS exemption claim be extended to include cut potatoes, as well as uses on page 3 of the notice and the information in section 6.3 be modified to include the new use. The use levels on page 11 would already encompass this additional use.

2. Please give us a little more information on the identity of the enzyme, if possible, for example, perhaps a molecular weight or residue count perhaps to more clearly define what the protein is.

From our perspective, you could send us a letter containing this information, indicating the sections or pieces of the notice that would be affected. You could send this electronically or by letter, or if you wish, you could do both. Feel free to call me at the number below if you have questions. Thanks. I'll let you know if we have additional questions or issues as they arise.

~~000115~~

000113

8/7/2006

Sincerely,

Bob Merker

Robert I. Merker, Ph.D.

Food and Drug Administration

Office of Food Additive Safety

Division of Biotechnology and GRAS Notice Review

Mailing Address:

5100 Paint Branch Parkway, HFS-255

College Park, MD 20740

Phone: 301-436-1226

FAX: 301-436-2964

e-mail: Robert.Merker@fda.hhs.gov

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~~XXXXXXXXXX~~



July 27, 2006

Robert Merker, Ph.D.  
Division of Biotech and GRAS Notice Review  
Office of Food Additive Safety  
Center for Food Safety and Applied Nutrition  
Food And Drug Administration  
5100 Paint Branch Parkway, HFS-255  
College Park, MD 20740-3835

Re: GRN 201

Dear Dr. Merker,

Novozymes' would like to clarify the intended uses of the asparaginase enzyme preparation produced by *Aspergillus oryzae* expressing the gene encoding an asparaginase from *Aspergillus oryzae* which is the subject of our GRAS notice No. 201. We realized that we were not consistent throughout our notice in listing all of the intended uses for the enzyme. We would like to make sure that the intended applications include the use in cut potatoes such as French fries and sliced potatoes as described on page 11 in our original notification. There is also a discussion of the use in French Fries in the Technology Sheet in Appendix 2.

We should have included this use in our cover letter, in the exemption claim document under "Applicable conditions of use" and in Section 1, Introduction, of the main notification document. The cover letter, and exemption claim should state, "The enzyme preparation is intended for use in the food industry as a processing aid to reduce formation of acrylamide in fabricated potato chips, wheat dough based snack foods such as cookies and crackers, and cut potatoes such as French fries and sliced potatoes.

In the Introduction on page 3 of the notification, the last sentence in paragraph 1 should say, "Asparaginase is intended to be used to convert asparagine to aspartic acid in order to reduce the formation of acrylamide in production of fabricated potato chips, wheat dough based products such as cookies and crackers, and cut potatoes such as French fries and slice potato products.

Finally, Section 6.3, Enzyme Residues in the Final Food should state:

The enzyme is added during the processing of snack type foods such as fabricated potato chips, wheat dough based products such as cookies, crackers and bread, and

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77 Perry Chapel Church Road  
P.O. Box 576  
Franklinton, North Carolina 27525

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NOVOZYMES

cut potatoes such as French fries. In dough based products, the enzyme is added to the dough before baking. In the fabricated potato chip application the enzyme is also added prior to a heat treatment or cooking step. In cut potato applications, the cut potatoes are soaked in an enzyme solution prior to par-frying and freezing. Frozen French fries are then subjected to an additional frying step before consumption. In each of these applications, the enzyme will be inactivated during the heat processing, cooking or frying treatments.

Please contact me by direct telephone at 919 494-3151, direct fax at 919 494-3420 or email at [lobg@novozymes.com](mailto:lobg@novozymes.com) if you have any questions or require additional information.

Sincerely,

(b)(6)

Lori Gregg  
Regulatory Specialist

000 116



AM

**Merker, Robert I**

**From:** LOBG (Lori Gregg) [LOBG@novozymes.com]  
**Sent:** Monday, August 07, 2006 10:48 AM  
**To:** Merker, Robert I  
**Subject:** RE: GRN 201 - more info on identity of enzyme

Dear Bob,

I hope you received my email of July 27 with the information in response to your #1 below. In response to your #2 below, I have had a chance to talk to my colleague in Denmark and we have the following information regarding the asparaginase enzyme which is the subject of GRN 201:

The molecular weight of the enzyme is approximately 36,000. It has an optimum pH of 7.0 and will work in the range of pH 5-8. It's optimum temperature is 60 C (at pH 7.0).

Please let me know if you have any questions.

Best Regards,

*Lori Gregg*  
Regulatory Specialist

**Novozymes North America, Inc.**  
77 Perry Chapel Church Road, Box 576  
Franklinton, NC 27525  
Phone: 919-494-3151  
Fax: 919-494-3420

-----Original Message-----

**From:** Merker, Robert I [mailto:robert.merker@fda.hhs.gov]  
**Sent:** Thursday, July 20, 2006 2:05 PM  
**To:** LOBG (Lori Gregg)  
**Subject:** long-delayed thoughts: GRN 201

Good afternoon, Ms. Gregg.

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1. That the uses in the cover letter and GRAS exemption claim be extended to include cut potatoes, as well as uses on page 3 of the notice and the information in section 6.3 be modified to include the new use. The use levels on page 11 would already encompass this additional use.
2. Please give us a little more information on the identity of the enzyme, if possible, for example, perhaps a molecular weight or residue count perhaps to more clearly define what the protein is.

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From our perspective, you could send us a letter containing this information, indicating the sections or pieces of the notice that would be affected. You could send this electronically or by letter, or if you wish, you could do both. Feel free to call me at the number below if you have questions. Thanks. I'll let you know if we have additional questions or issues as they arise.

8/7/2006

Sincerely,

Bob Merker

Robert I. Merker, Ph.D.

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8/7/2006