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December 3, 2013

Via FedEx

Food and Drug Administration
Division of Animal Feeds (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: CVM GRAS Notification for L-Methionine From a Modified *Escherichia coli* K-12; Our File No. RO02856-24

Dear Sir or Madam:

On behalf of our client, Roquette Frères (the Notifier), we respectfully submit the attached Notification in support of the determination that L-methionine produced by a genetically modified *Escherichia coli* K-12 is Generally Recognized as Safe (GRAS) when used as a nutrient at levels up to 0.3% in animal feed. The enclosed Notification is a resubmission of the filing that was submitted on Roquette's behalf on April 4, 2013. The prior filing was withdrawn on May 3, 2013 to provide additional time to respond to questions and comments raised by the Food and Drug Administration in its preliminary review. A meeting was held on June 10, 2013 to discuss these questions and comments in more detail. We believe the enclosed Notification now addresses these questions and comments.

Roquette Frères has determined that L-methionine produced by a genetically modified *E. coli* K-12 is GRAS based on scientific procedures in accordance with 21 C.F.R. § 570.30(b). The enclosed GRAS Notification provides a review of the information related to the intended uses, manufacturing, and safety of the ingredient. The analytical data, published studies, and information that are the basis for this GRAS determination are included with this Notification. We have included three (3) hard copies of the GRAS Notification and its Attachments. All cited references are included on the CD included with each copy of the Notification.

Please note that portions of the Notification and the whole of Attachment 4 contain trade secret and confidential business information, which should not be disclosed to the public in accord with the Agency's public information regulations under 21 C.F.R. Part 20. The information that we claim as exempt from disclosure is marked "CONFIDENTIAL" in the

Notification. If you disagree with our confidential claims, we respectfully request that the Agency notify us, in accordance with Section 20.61(e)(1), prior to any release of the Notification on FDA's website or otherwise.

We trust that this submission satisfies the Agency's needs, and will be deemed accepted and complete. Should any questions arise, please contact us, preferably by telephone or e-mail, so that we can promptly respond.

Sincerely yours,

Melvin S. Drozen

Enclosures

Generally Recognized as Safe (GRAS) Notification

for

L-Methionine 85% From a Modified *Escherichia coli* K-12

Prepared for:
U.S. Food and Drug Administration
Center for Veterinary Medicine
Division of Animal Feeds (HFV-224)
7519 Standish Place
Rockville, MD 20855

Prepared by:
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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

ATTACHMENTS

I. INTRODUCTION

This notification has been prepared by Keller and Heckman LLP on behalf of Roquette Frères, and is submitted in support of the determination that L-methionine produced by a genetically modified *Escherichia coli* K-12 is Generally Recognized as Safe (GRAS) when used as a nutrient for animal consumption. Under 21 C.F.R. § 582.5475, methionine is considered GRAS when used in accordance with good manufacturing or feeding practice. Methionine exists as a stereoisomer, either as D-methionine or L-methionine. The regulation does not identify that a specific stereoisomer is the only methionine covered and L-methionine is the physiologically relevant stereoisomer. Accordingly, 21 C.F.R. § 582.5475 includes L-methionine, which is therefore considered GRAS by FDA. As discussed further below, *E. coli* K-12 is a non-pathogenic, non-toxigenic bacterial strain – which FDA considers to be a safe production organism – that has been modified to over-express endogenous genes involved in the methionine biosynthetic pathway. Because *E. coli* K-12 is considered a safe production organism and only endogenous genes were inserted into the strain, any impurities arising from the *E. coli* K-12 production strain remaining with the L-methionine after fermentation and purification are considered safe.

As Eric Flamm indicates in "How FDA Approved Chymosin: A Case History," **Attachment 1**, evidence that *E. coli* K-12 is nonpathogenic and nontoxigenic, and therefore safe, includes studies showing that *E. coli* K-12 does not colonize the gut of man or other animals even at high concentrations (10^9 to 10^{10} viable organisms per ingestion),[1] that the K-12 strain has been widely used as a laboratory organism for 30 years with no reported incidents of illness,[2] that it does not produce toxins that cause illness upon ingestion,[3] and that it is deficient in

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1 Gorbach, S.L., "Recombinant DNA: An infectious Disease Perspective," Journal of Infectious Diseases 137:615-623 (1978); Curtiss, R., "Biological Containment and Cloning Vector Transmissibility," Journal of Infectious Diseases 137:668-675 (1978); and Smith, H.W., "Is it Safe to Use *Escherichia coli* K-12 in Recombinant DNA Experiments?" Journal of Infectious Diseases 137:655-660 (1978).

2 *See* Gorbach (1978), footnote 1.

3 *See* Gorbach (1978) and Curtiss (1978), footnote 1.

virtually all characteristics necessary for pathogenesis.[4] Additionally, non-pathogenic strains of *E. coli* are a part of the normal flora of the gastrointestinal tract of man, where they are found at 10^6 to 10^8 organisms per gram of intestinal contents.[5]

The determination of GRAS status is on the basis of scientific procedures and conforms to the guidance issued by the Food and Drug Administration (FDA) under *proposed* 21 CFR § 570.36, 62 Fed. Reg. 18938 (Apr. 17, 1997) and the FDA's Notice of Pilot Program; Substances Generally Recognized as Safe Added to Food for Animals, 75 Fed. Reg. 31806 (June 4, 2010). We submit information in the following areas:

- Identity of the substance;
- A description of the method of manufacture;
- Safety data and safety evaluation; and
- Determination that the impurities arising from the modified *E. coli* are safe.

It is our expectation that FDA will concur that the information presented here fully supports the determination that L-methionine produced by a genetically modified *Escherichia coli* K-12 is Generally Recognized as Safe.

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4 *See* Gorbach (1978), footnote 1.

5 *Id.*

II. ADMINISTRATIVE INFORMATION

A. CLAIM OF GRAS STATUS

The submitter has determined that the use of L-methionine 85%, produced by a genetically modified *Escherichia coli* K-12, for use as a nutrient in animal feed is Generally Recognized as Safe based on scientific procedures, and is thus exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 301 *et. seq.*)(the Act).

Mel Drozen 12/3/13

Signature Date

B. NAME AND ADDRESS OF THE SUBMITTER

Submitter

François Creton
Regulatory Affairs Manager
Roquette Frères
Batiment Alpha 3
1 rue de la haute loge
62136 Lestrem FRANCE

Acknowledgement of Receipt of Notification and Inquiries to be Directed to:
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C. COMMON OR USUAL NAME OF THE SUBJECT SUBSTANCE

The common or usual name of the subject substance of this notification is L-methionine 85%, also known as methionine 85%. The product will be identified and sold as "L-methionine 85%." The finished nutrient produced by the genetically modified *E. coli* has an L-methionine content of 85%. Because this is a lower L-methionine content than required by organizations such as the Association of American Feed Control Officials (AAFCO) and the U.S. Pharmacopeia (USP), the product is being identified with its L-methionine content directly in the name to differentiate it.

D. INTENDED CONDITIONS OF USE AND TECHNICAL EFFECT

The L-methionine 85% is to be used as a nutrient in animal feeds in accordance with current good manufacturing or feeding practice as defined in 21 C.F.R. § 582.1(b) ("Substances that are generally recognized as safe"). L-methionine is an essential amino acid in all animal species. The level of supplementation that is safe varies between species and is dependent on the basal diet and its amino acid content. Therefore, the required level of supplementation will be determined on a case-by-case basis by animal nutritionists or veterinarians, based on good feeding practice for the target species.

E. BASIS FOR THE GRAS DETERMINATION

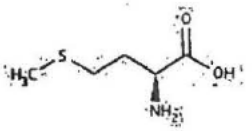
Pursuant to 21 C.F.R. 570.30(a)(1), scientific procedures were used to establish that the modifications made to the *E. coli* strain to produce methionine does not alter the GRAS status of L-methionine, and the impurities arising from the use of the modified *E. coli* K-12 are safe.

F. AVAILABILITY OF INFORMATION

At the request of the Center for Veterinary Medicine, the submitter has included copies of the scientific data and information/references that form the basis for the GRAS determination. Copies of the references are provided on the CD included with this submission.

III. DETAILED INFORMATION ABOUT THE IDENTITY OF THE NOTIFIED SUBSTANCE

A. NAME AND OTHER IDENTITIES

Chemical Name:	L-methionine
CAS Registry Number:	63-68-3
Molecular Formula:	$C_5H_{11}NO_2S$
Structure	

Other names:	(S)-2-Amino-4-(methylthio)butanoic acid; 2-Amino-4-(methylthio)butyric acid; 2-Amino-4-methylthiobutanoic acid; Acimethin; Butanoic acid, 2-amino-4-(methylthio)-, (S)-; Cymethion; h-Met-oh; L-Homocysteine, S-methyl-; L-α-Amino-γ-methylthiobutyric acid; S-methionine; S-Methyl-L-homocysteine; α-Amino-γ-methylmercaptobutyric acid; γ-Methylthio-α-aminobutyric acid

L-Methionine is an essential amino acid that is required in the diets of humans and animals to maintain health.

The intended use of L-methionine 85% is as a nutrient at levels consistent with good feeding practice. Methionine is recognized as GRAS under 21 C.F.R. § 582.5475 when used in accordance with current good manufacturing or feeding practice. Methionine exists as a stereoisomer, either as D-methionine or L-methionine; L-methionine is the physiologically relevant stereoisomer. 21 C.F.R. § 582.5475 does not identify a specific stereoisomer as the only structure covered, thus, L-methionine can be considered GRAS. Because L-methionine is recognized as GRAS in animal feed, the only additional relevant determination is the safety of the impurities arising from production using the modified *E. coli* K-12. Further information on this is provided below.

B. COMPOSITION/SPECIFICATIONS

The majority of the product is free L-methionine (≥85 %). The product also consists of other amino acids, (b) (4). Notifier has sponsored complete analyses of three lots of L-methionine 85%. Specific analytes included total amino acids (2.3%), (b) (4) . As displayed in the comprehensive tables provided in **Attachment 2-A** and **Attachment 2-B**, the final product was found to contain approximately (b) (4); addition of all the analytes described

above resulted in approximately the same amount of material, including common metabolites such as (b) (4), and common metabolic products of amino acids and proteins.

An approximate material balance is provided in the following table.

Table 1: Approximate Material Balance for L-Methionine 85%*

Analyte	Percentage (%)
(b) (4)	
Total	98.7%

*Based on the data provided in Attachment 2-B[6]

Based on the analyses of Methionine 85% we conclude that this product is substantially equivalent to DL-methionine for the purpose of feed supplementation in the target animals. No component of L-methionine 85% differs significantly from the normal constituents of the ordinary diet of the target animals. The safety of potential exposures to these substances is discussed further in Section IV.A.3.

The major components defined by specifications for the product are provided in the following table.

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6 For substances that were not detected, values of one-half of the limit of detection were used. Amino acids was rounded to (b) (4) to account for (b) (4) Common metabolites refers to the biogenic amines and other organic compounds.

Table 2: Specifications

L-Methionine	minimum 85%
Loss on drying	maximum 3%
Ash	maximum 3%
Fat	maximum 0.2%
Residual sugars	maximum 0.5%

To demonstrate compliance with the specifications, three (3) Certificates of Analysis (COAs) and associated analytical methods are included as **Attachment 3**.

(b) (4)

C. MANUFACTURE

The general production process of L-methionine 85% is shown in Figure 1 and discussed in further detail below.

Figure 1: L-Methionine Manufacture/Purification Diagram

(b) (4)

1. Fermentation

Fermentation process begins with (b) (4) , and the equipment is regularly cleaned and maintained to prevent microbial contamination. Antibiotics are not used during the manufacturing processing (fermentation and post-fermentation processing).

a) Precultures

Laboratory precultures are in flasks or small fermentors under controlled temperature and agitation. Each preculture has specific processing parameters such as growth medium, volume, inoculum, time and temperature treatment as provided below in Table 3 and specific growth media components provided in

Table 4. Technical data sheets (TDS) and material safety data sheets (MSDS) are provided for each component of the growth media in **Attachment 4**.

(b) (4)

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b) **Production**

The fermentation is carried out in a fermentor with a capacity of (b) (4) under controlled and optimal (b) (4) to meet the requirements of the strain. Table 5 below describes the process parameters used during the production stage with specific growth media components provided in Table 6.

(b) (4)

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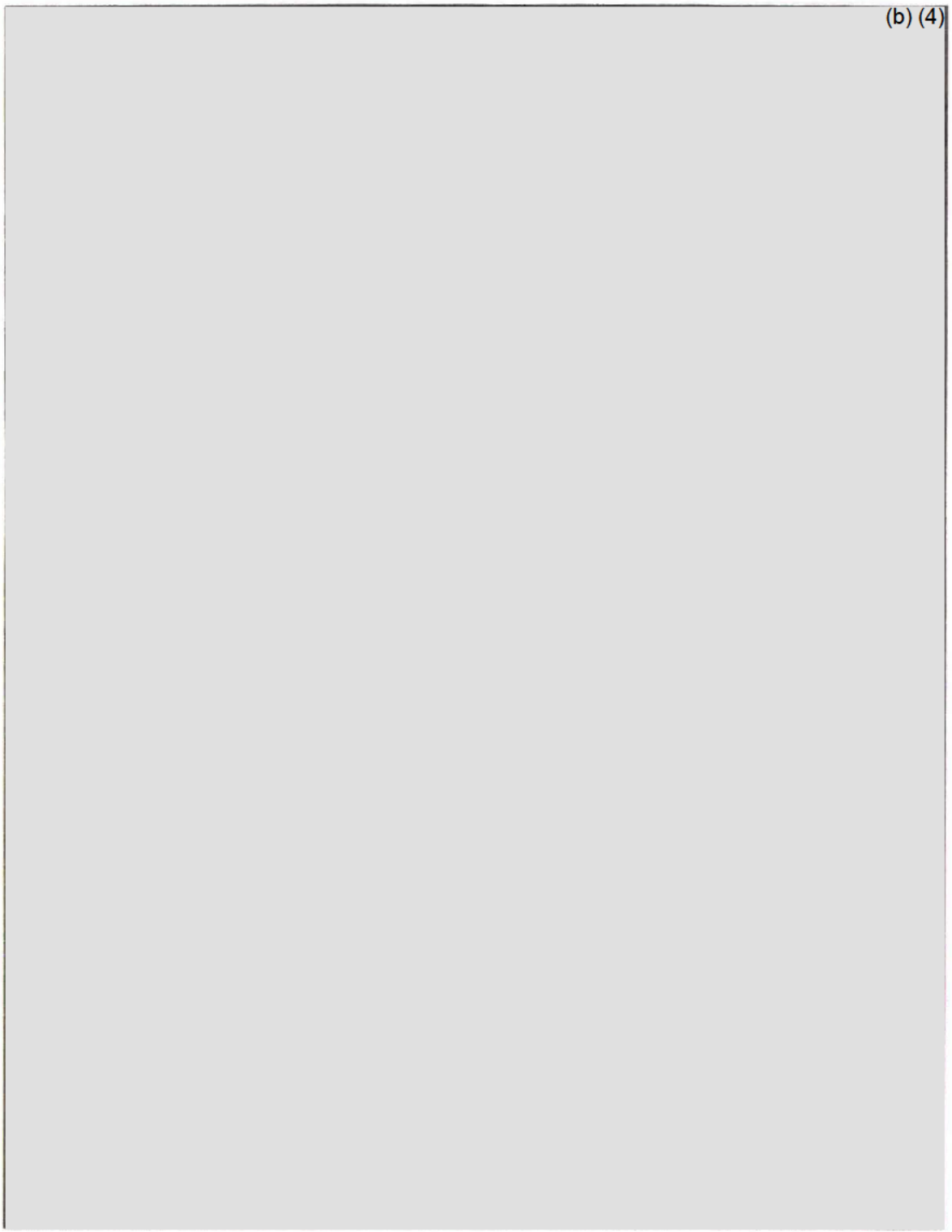
(b) (4)



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(b) (4)

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The final product is analyzed according to the specifications as described in Section III. B., Composition/Specifications.

D. INFORMATION ON ANY SELF-LIMITING LEVELS OF USE

The self-limiting levels of use are those associated with current good manufacturing and good feeding practices for L-methionine, as determined based on the species to which the L-methionine is being provided, the animal's current protein and amino acid intake, the limiting amino acid, the age of the animal, and other applicable factors.

When formulating diets for food animals with commonly available grains and protein sources, the protein may or may not contain adequate amounts of amino acids to meet the

animal's requirements, based on a variety of factors.[7] If a diet is inadequate in any essential amino acid, protein synthesis cannot proceed efficiently. The 10 essential amino acids that must be provided in the diets of all animals, including humans, are: lysine, threonine, tryptophan, methionine (and cystine), isoleucine, histidine, valine, arginine, phenylalanine, and tyrosine. Methionine is recognized as the first limiting amino acid in poultry, in high-yielding cows, and as the second or third limiting amino acid in pigs fed conventional diets.[8] Methionine is essential for maintaining proper growth and development in mammals; and its supplementation in domestic animals, such as chicks and pigs, contributes to better production efficacy.[9] Therefore, the use of L-methionine 85% will be limited to the level appropriate for the specific animal to maximize growth and development.

As described above, L-methionine is GRAS under 21 C.F.R. § 582.5475 when used in accordance with current good manufacturing or feeding practice. Consequently, all of the L-methionine products currently on the market are being used at appropriate levels for the target animals without a regulatory limitation. Because the L-methionine produced using the *E. coli* K-12 is the same amino acid, and because the risk assessment for the impurities present in the product, as described in Section IV below, demonstrates the safety of the product, L-methionine 85% produced as described in this Notice should similarly be considered GRAS when used in accordance with current good manufacturing or feeding practice.

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7 The public literature contains many articles relating to the variation of amino acid requirements based on species, the existing diet, age of the animal, and limiting amino acid. For example, see Swine Nutrition Guide: Prepared by Hansen, J.A., National Swine Nutrition Guide Tables on Nutrient Recommendations, Ingredient Composition, and Use Rates (2010), U.S. Pork Center of Excellence, available at http://www.ncsu.edu/project/swine_extension/nutrition/nutritionguide/default.htm.

8 EFSA, *Scientific Opinion on DL-methionine, DL-methionine sodium salt, the hydroxy analogue of methionine and the calcium salt of methionine hydroxy analogue in all animal species; on the isopropyl ester of methionine hydroxy analogue and DL-methionine technically pure protected with copolymer vinylpyridine/styrene in dairy cows; and on DL-methionine technically pure protected with ethylcellulose in ruminants*, EFSA Journal 10(3), 2623-2664 (2012).

9 Chung, T.K. and Baker, D.H., "Methionine requirement of pigs between 5 and 20 kilograms body weight," J. Anim. Sci., 70: 1857–1863 (1992); Meirelles, H.T. *et al*, "Performance of broilers fed with different levels of methionine hydroxy analogue and DL-methionine," Rev. Bras. Cienc. Avic., 5(1) (Jan/Apr 2003), available at http://www.scielo.br/scielo.php?pid=S1516-635X2003000100009&script=sci_arttext.

E. SAFETY OF *ESCHERICHIA COLI* K-12

Escherichia coli is a naturally occurring, Gram-negative intestinal inhabitant for both humans and animals.[10] Although some strains of *E. coli*, such as O157:H7, are pathogenic, *E. coli* K-12 strains are non-pathogenic and often serve as a benign reference model to understand the mechanisms of pathogenicity in pathogenic strains of *E. coli*.[11] *E. coli* K-12 was selected for its lack of pathogenicity and has been maintained in a laboratory setting since 1922[12] with no reports of *E. coli* K-12 infections.[13] *E. coli* K-12 does not produce Shiga-like toxins that are produced by pathogenic *E. coli* strains.[14] *E.* coli K-12 is considered to be one of the most well-studied microbes, and the genome of *E. coli* K-12 was sequenced in 1997.[15] *E. coli* K-12 is classified by the National Institutes of Health (NIH) to be a Class 1 Agent, which means that it is not considered to be either a human or animal pathogen.[16]

E. coli K-12 has been described as a "crippled organism"[17] with an established history of safe use for the commercial production of various products through recombinant DNA technology.[18] In fact, the first recombinant-enzyme product approved for use in food by FDA in 1991 was the enzyme bovine chymosin, used in the production of cheese, which was also expressed in *E. coli* K-12. As stated on page 349 in Eric Flamm's 1991 article on the FDA approval of chymosin, Attachment 1, "FDA based its conclusion that the production strain is safe

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10 Smith, W., "A search for transmissible pathogenic characters in invasive strains of *Escherichia coli*: the discovery of a plasmid-controlled toxin and a plasmid-controlled lethal character closely associated, or identical, with colicine V," Journal of General Microbiology 83:95-111 (1974); Olempska-Beer, Z.S., et al, "Food-processing enzymes for recombinant microorganisms – a review," Regulatory Toxicology and Pharmacology 45:144-158 (2006).

11 Hayashi T., et al, "Complete genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison with a laboratory strain K-12," DNA Research 8:11-22 (2001).

12 *See* Curtiss (1978), footnote 1.

13 *See* Olempska-Beer (2006), footnote 10.

14 *Id.*

15 *Id.*

16 *See* "*Escherichia coli* K-12 derivatives final risk assessment" from the U.S. Environmental Protection Agency found at http://www.epa.gov/oppt/biotech/pubs/fra/fd004.htm (Last Accessed March 20, 2012).

17 *See* Smith (1978), footnote 1.

18 *See* Hayashi (2001), footnote 11.

for use in the production of a purified chymosin preparation primarily on published evidence demonstrating that it is nonpathogenic and nontoxigenic." The article continues, "even though it is not a common food-use organism and there have been no traditional feeding studies performed with it, FDA concluded that there is sufficient published information on *E. coli* K-12 to demonstrate that it is safe for producing chymosin." Based on the available data, FDA concluded that *E. coli* K-12 is a nonpathogenic and nontoxigenic organism that is considered safe for producing the non-endogenous protein, bovine chymosin.

Based on FDA's published determination that *E. coli* K-12 is a safe production organism, and the published studies in chickens, calves, and rabbits, it can be concluded that *E. coli* K-12 is a nonpathogenic and nontoxigenic strain to animals and is, therefore, a safe production organism.

F. SAFETY CONSIDERATIONS DUE TO THE NATURE OF MODIFICATIONS TO *E. COLI*

Below we address the genetic construction and anticipated metabolic pathway of the *E. coli* K-12 cell. Although the product of these processes, L-methionine 85%, is an essential amino acid and not an enzyme, we have conservatively evaluated the safety of the production organism using the Pariza and Johnson decision tree, as demonstrated in **Attachment 5.**[12] This decision tree is intended primarily to provide guidance for evaluating the safety of enzyme preparations used in animal feed; nevertheless, we consider the questions posed in the decision tree to be relevant to demonstrate the safety of L-methionine 85% in animal feed. As described in more detail below, because the modified production strain is from a safe and non-pathogenic *E. coli* K-12 lineage, contains inserted coding sequences that are well characterized and endogenous, the final commercial strain is free from antibiotic resistance genes, and because the final L-methionine 85% has been determined to be free of toxins and other unsafe metabolites, we conclude that the genetic modifications to the *E. coli* and the resulting production strain are acceptable and safe.

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12 Pariza, M.W. and Cook, M., "Determining the safety of enzymes used in animal feed," Regul. Toxicol. Pharmacol. 56(3):332-42 (Apr. 2010). The decision tree is included as Attachment 5.

1. Genetic Description

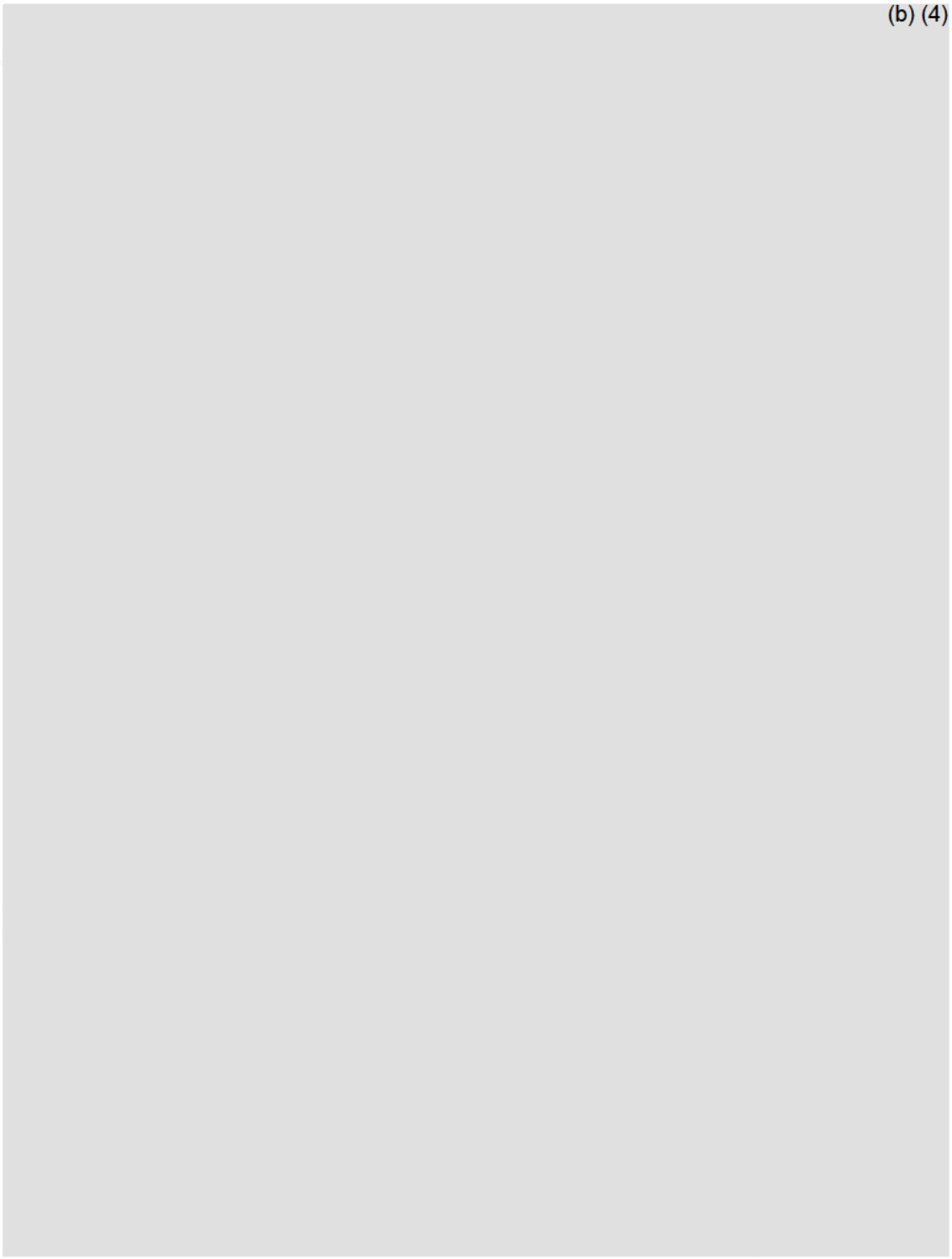
The parental *E. coli* K-12 cell is (b) (4), which is identified as *E. coli* K-12 (b) (4)[20] All coding sequences inserted are endogenous to *E. coli* K-12. Please refer to Figure 2 for a diagram of the methionine pathway genetic modifications. The modifications were performed using (b) (4) . The inserted sequences were all chromosomally integrated, which is generally recognized to be genetically stable and therefore poorly transmissible. (b) (4) were inserted or genetically modified in the final production strain. Promoters used have no known adverse effects and an established history of use, and several of the genes are under the control of their native promoters. Variations in the gene expression patterns of *E. coli* K-12 genes often occur naturally due to genetic variation between *E. coli* strains and environmental stimuli, and as *E. coli* K-12 has an established history of safe use, the modifications to the expression patterns of endogenous genes is not anticipated to be a hazard.

The final strain has the following genotype:

(b) (4)

[20] http://www.genome.wisc.edu/resources/strains.htm.

(b) (4)



(b) (4)

[redacted]

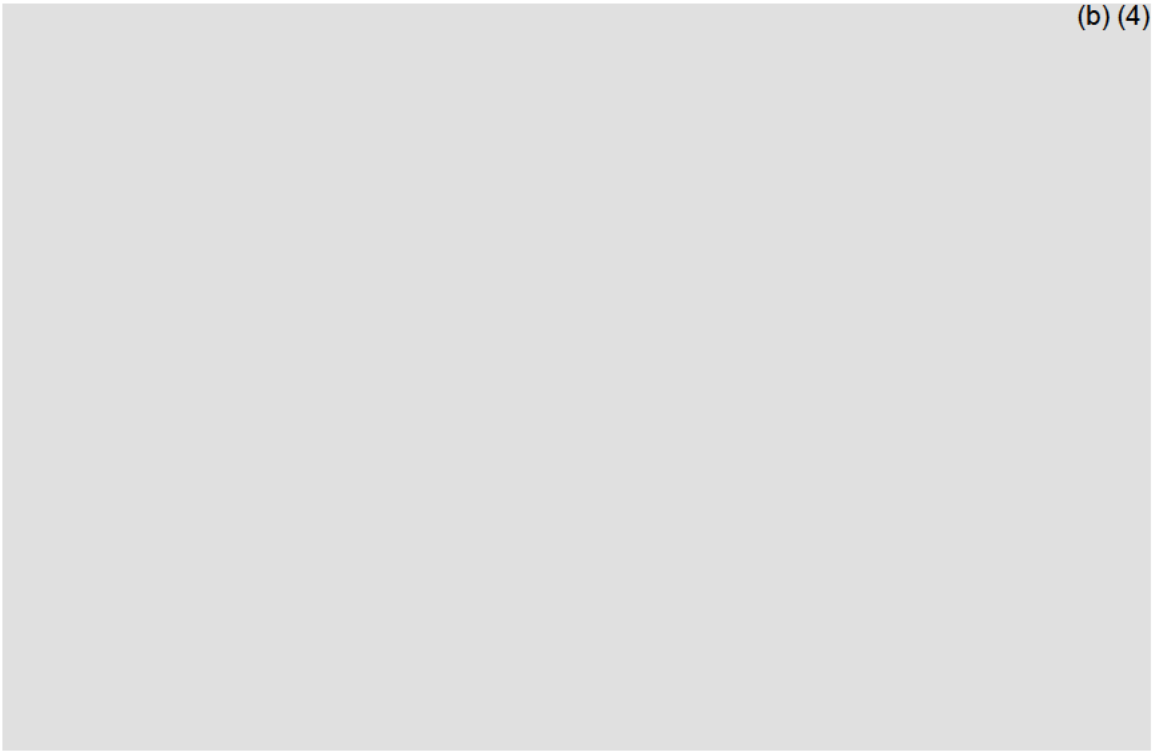
(b) (4)

[redacted]

(b) (4)

[redacted]

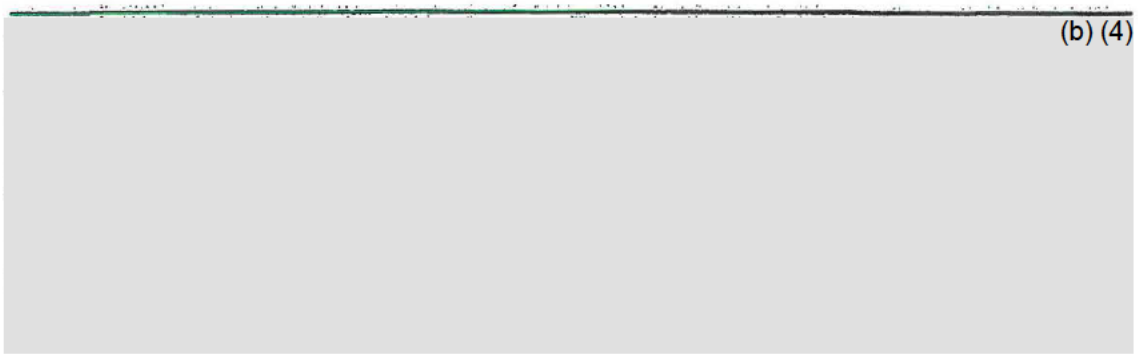
(b) (4)



2. Anticipated Metabolic Pathway

The purposes of the inserted genes are: (1) for inactivation of the feedback loops and production loops in the endogenous pathway; and (2) to increase methionine production as shown in Figure 2. All insertions were integrated directly into specific gene loci within the chromosomal DNA as described above using endogenous gene sequences.

(b) (4)



(b) (4)

Since the modified pathway is an endogenous pathway, the intermediate metabolites in the pathway are naturally present in *E. coli* K-12.

The metabolic efficiency of the modified *E. coli* K-12 to produce methionine is shown in the complete compositional analysis (Attachment 2). As shown in the analysis, the majority of the L-methionine 85% is, as expected, methionine and minor or trace amounts of other amino acids, (b) (4), etc.

3. Genetic Construction

The chronological order of the insertions and deletions are provided in Figure 3 below. Genetic modifications were performed using homologous recombination through the transformation vector (b) (4) into an *E. coli* K-12 strain (b) (4) is a commonly used transformation vector[43] and general information on the vector can be accessed at (b) (4). The genetic modifications were then introduced through transduction and homologous recombination into the final production strain, which was also an *E. coli* K-12 strain (b) (4). All inserted genes were chromosomally integrated. As the gene expression for (b) (4) in Figure 3), so that the final commercial strain has (b) (4) gene in the (b) (4) construct.

Deletion means excision of one (ex: (b) (4) or several genes (ex: (b) (4); "PM" stands for promoter modification; "Replacement" indicates insertion of a coding sequence into a deleted locus. (b) (4) was subsequently removed and completely replaced by (b) (4) as described above and in Figure 3, below.

(b) (4)

(b) (4)

G. ALLERGENICITY

No foreign proteins were inserted into the strain, only endogenous proteins, which will not be present in the final product at detectable levels.

H. REMOVAL OF ALL ANTIBIOTIC RESISTANCE MARKERS

The final commercial production strain does not contain any antibiotic resistance markers. However, during the construction of the modified *E. coli* strain, (b) (4)

(b) (4)

(b) (4)

I. GENETIC INSERTION SITES

All inserted genes were performed through well characterized (b) (4) and therefore the genes were not randomly inserted. Insertion locations were verified through PCR analysis as provided in **Attachment 6.**

Table 8: Summary of insertion sites

(b) (4)

(b) (4)

[redacted]

J. COPY NUMBERS GENE SOURCE

As described above in the section entitled "genetic modifications" several of the genes are present in multiple copies. Table 9 below summarizes the genes present in more than one copy for purposes of increasing protein expression in the (b) (4) production strain.

Table 9: Summary of inserted gene copy numbers

Gene	Inserted Copy Numbers[#]	Natural locus copy number	Total copy number
(b) (4)			

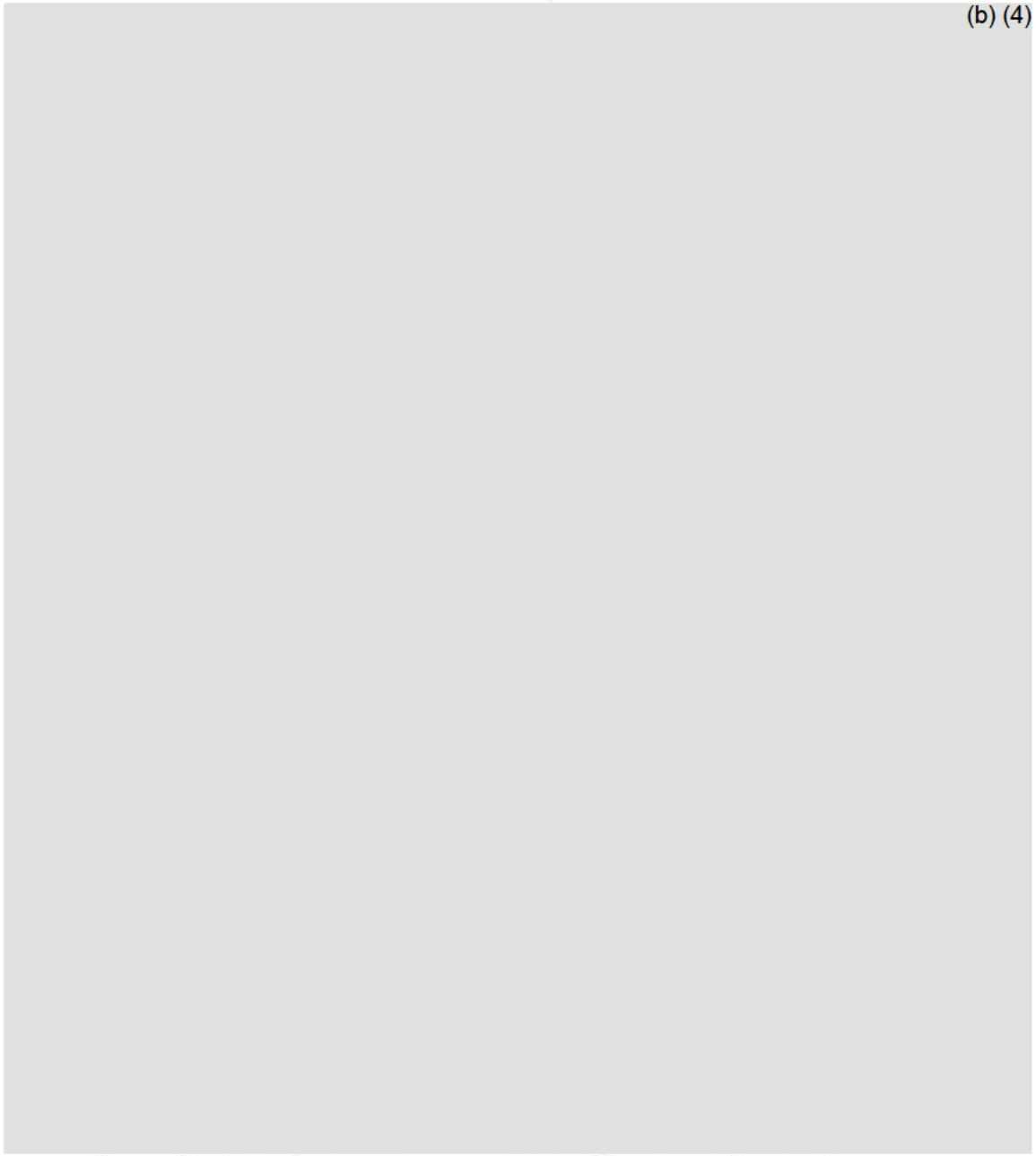
All inserted genetic material, which is listed in the above Table 9, was directly PCR cloned from *E. coli* K-12.

K. TRANSFORMATION PLASMIDS

1. (b) (4) Plasmid

(b) (4)

(b) (4)



L. ALTERATIONS IN METABOLITE LEVELS (SPILL OVER EFFECTS)

It is our understanding that genetic modifications can alter the metabolite concentrations within the host organism, which may lead to increased exposure to metabolites of concern. However, for the following reasons, we believe that the use of L-methionine 85% derived from a

modified *E. coli* K-12 is unlikely to cause exposure to such effects in either animals or humans consuming animal tissue and products:

1) *E. coli K-12* is well characterized and is not known to produce any metabolites of concern, such as toxins. In addition, it has an established safe history of use as a source of enzymes used in the food industry;

2) All the genetic modifications were performed on endogenous genes, either by deletion or insertion of extra copies for purposes of overexpression of the metabolic enzymes. Therefore, the metabolic pathway is well characterized (see **Figure 2**) and the likelihood of unintended effects is reduced compared to the insertion of a foreign protein;

3) The strain has been modified to maximize conversion of resources toward L-methionine production through strategic overexpression of key enzymes in the L-methione pathway and deletion of by-product pathways. This reduces the concentration of unwanted metabolites; and

4) Target animals will consume only a highly purified L-methionine product, not the entire *E. coli* K-12, and, as described in Attachment 2, the L-methionine product is well characterized. The residues that are not amino acids make up approximately 12% of the final product, with each component at a very low concentration (see risk assessment of metabolites in Section IV.C. below).

Given the above descriptions and data discussed above, we have no reason to believe that under its intended conditions of use L-methionine 85% is not safe. However, we have performed an analysis of the "spill–over effects" to further support the product's safety.

All amino acids share a common structure that contains an asymmetric alpha carbon, a hydrogen, an amino group, a carboxyl group, and a variable group, which is the only group that varies between amino acids. Given this common structure between amino acids, it is not surprising to find that many metabolic pathways for other amino acids intersect with the L-methionine pathway (See **Figure 2**). For example, as illustrated in **Figure 2**, the amino acid

(b) (4)

(b) (4). In general, as presented in **Attachment 2**, the levels of the non-methionine amino acids are significantly lower, with the highest level at 0.76% detected for (b) (4). These amino acids are a common and important component of the animal diet. For example, distillers grains, which is commonly fed to livestock and companion animals at rates significantly higher rates (15-30%[47]) as compared to L-methionine, have an average amino acid profile for the non-L-methionine amino acids at higher percentages than those found in this product.[48] Therefore, the concentrations of non-methionine amino acids in the L-methionine product are safe.

The biogenic amines measured in **Attachment 2**, namely cadaverine, spermidine and putrescine, are known to be normal metabolic breakdown products of amines such as amino acids. Since the genes involved in the production of cadaverine, spermidine, and putrescine, namely *speA* (arginine decarboxylase), *sepB* (agmatine ureohydrolase), and *speC* (ornithine decarbosylase), and *speD* (adenosylmethionine decarboxylase),[49] were not genetically modified in the *E. coli* production strain, the levels of these biogenic amines are not expected to be altered. Regardless, the levels of these biogenic amines measured in the finished L-methionine product

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47 http://www.distillersgrains.org/feedsource/.

48 Liu, K., "Chemical Composition of Distillers Grains, a Review," Journal of Agricultural and Food Chemistry 59: 1508-1526 (2011).

49 Tabor, H., Hafner, E., and Tabor, C., "Construction of an *Escherichia coli* strain unable to synthesize putrescine, spermidine, or cadaverine: characterization of two genes controlling lysine decarboxylase." Journal of Bacteriology 144(3): 952-956 (1980).

were insignificant. (**Attachment 2**; (b) (4))

The organic acids found in the final L-methionine product are also common and normal cell metabolites from the metabolism of carbon compounds, such as (b) (4) For example, one

(b) (4)

The other organic compounds measured in the finished L-methionine product were all identified to be normal cell metabolites (see Table 11, Residual analysis of final product) and when measured (**Attachment 2**) found to be at insignificant levels (the highest reported level was at 0.73% of the finished L-methionine product with the majority of the other compounds at significantly lower levels).

Therefore, given that the production organism *E. coli* K-12 is not known to produce toxins or other metabolites of concern, genetic modifications were performed in only endogenous genes and intentionally engineered to divert the maximum amount of metabolites towards L-methionine production, and the finished L-methionine product is purified and well characterized, we conclude that the genetic modifications will not result in the exposure of metabolites of concern at levels of concern to target animals fed the L-methionine product.

M. STABILITY TESTING

Roquette conducted two separate tests to demonstrate the stability of the L-methionine 85% under the likely storage conditions. Both studies were conducted using the same three batches of L-methionine 85%. The report of this study is included as **Attachment 7**.

(b) (4)

1. Analytical methods

L-Methionine: See Annex III, " (b) (4)

(b) (4)

(b) (4)." The variability of the method is ± 8%. The possible range for a product meeting the specification for methionine content reported as 85% by weight would be 77-93%. The analysis was conducted on production batches. Any batch that did not meet the L-methionine 85% specification would not be acceptable for release. However, for the purpose of reporting and comparing composition, the batches provide reliable data.

(b) (4)

2. Study 1:

Packaging: (b) (4)

Bags held at 25°C with 60% relative humidity for 24 months:

Analyte	Batch #	Time (months)						
		T0	T1	T3	T6	T12	T18	T24
L-Methionine	1	83.67	81.18	82.95	87.43	83.24	84.41	85.12
	2	84.35	81.47	82.63	86.23	82.81	84.73	84.67
	3	82.99	81.54	82.8	86.8	84.18	85.52	84.63
(b) (4)	1							(b) (4)
	2							
	3							

Bags held at 40°C with 75% relative humidity for 18 months:

Analyte	Batch #	Time (months)				
		T0	T1	T3	T6	T18
L-Methionine	1	83.83	82.28	83.34	87.49	85.15
	2	84.38	81.28	83.42	86.99	84.64
	3	83.58	82.08	83.67	87.84	84.91
(b) (4)	1					(b) (4)
	2					
	3					

3. Study 2:

Packaging: (b) (4)

Bags held at 25°C with 60% relative humidity for 24 months:

Analyte	Batch #	Time (months)						
		T0	T1	T3	T6	T12	T18	T24
L-Methionine	1	82.6	85.05	84.93	84.57	85.25	83.96	84.3
	2	83.13	85.12	86.17	84.48	84.48	83.68	83.0
	3	83.61	85.56	86.33	85.5	85.65	83.59	84.30
(b) (4)	1							(b) (4)
	2							
	3							

Bags held at 40°C with 75% relative humidity for 18 months:

Analyte	Batch #	Time (months)				
		T0	T1	T3	T6	T18
L-Methionine	1	84.06	84.6	84.97	83.1	81.7
	2	83.28	84.46	84.36	82.41	81.8
	3	83.47	84.44	85.36	82.99	82.41
(b) (4)	1					(b) (4)
	2					
	3					

IV. DETAILED SUMMARY OF THE BASIS FOR THE GRAS DETERMINATION

A. ANIMAL SAFETY ASSESSMENT FOR *E. COLI* K-12

General background information regarding *E. coli* K-12 and safety assessments are provided in section III.E above entitled "Safety of *Escherichia coli* K-12."

1. Chickens

E. coli K-12 safety in chickens was shown by the intravenous injection studies of *E. coli* K-12 modified to express colicin V (ColV) and other plasmids from enterobacterial strains. ColV is a virulence factor often found in strains of *E. coli* pathogenic to birds.[51] The 1974 study

[51] Johnson, T., Siek, K., Johnson, S., and Nolan, L., "DNA Sequence of a ColV Plasmid and Prevalence of Selected Plasmid-Encoded Virulence Genes among Avian *Escherichia coli* Strains," Journal of Bacteriology 188:2, 745-758 (January 2006).

by Smith[52] shows that *E. coli* K-12 is nonpathogenic and nontoxigenic in chickens, but becomes pathogenic and toxigenic when ColV was expressed. Unmodified *E. coli* K-12 and *E. coli* K-12 modified with non-ColV plasmids did not show high mortality, whereas *E. coli* K-12 modified with ColV did show high mortality. Based on this study, *E. coli* K-12 is nonpathogenic and nontoxigenic in chickens.

2. Cattle

E. coli K-12 safety in cattle was shown through feeding studies of *E. coli* K-12 modified to contain the pathogenic plasmids K99 and Ent (enterotoxin). The K99 plasmid has been shown to increase the ability of *E. coli* to proliferate in the small intestines, and the Ent plasmid causes diarrhea.[53] The 1978 paper by Curtiss[54] showed that when four colostrum-deprived calves (which lack bacterial antibodies and are more vulnerable to bacterial infections) were fed *E. coli* K-12 modified with a combination of K99 and Ent plasmids, one calf displayed mild diarrhea and the other three had no significant symptoms. When the small intestines of the calves were examined, the modified *E. coli* K-12 had not undergone detectable proliferation. A fifth calf was given an enteropathogenic *E. coli* strain, the *E. coli* K-12 modified strain, and a non-pathogenic *E. coli* strain. That calf developed severe diarrhea and was nearly dead in 30 hours. A bacterial examination showed high concentrations of the enteropathogenic strain, low concentrations of the non-pathogenic *E. coli* strain, but none of the modified *E. coli* K-12 strain.

Another feeding study by Falkow *et al.* (1976)[55] also showed that *E. coli* K-12 was safe. In the study, calves were fed either *E. coli* K-12 without the Ent plasmid or *E. coli* K-12 modified with the Ent plasmid. There were no reports of illness or significant symptoms reported in any of the calves fed either the *E. coli* K-12 without the Ent plasmid or the *E. coli* K-12 with the Ent plasmid.

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52 *See* Smith (1978), footnote 1.

53 *See* Curtiss III (1978), footnote 1.

54 *Id.*

55 Falkow, S., Williams Jr., L., Seaman, S., and Rollins, L., "Increased survival in calves of *Escherichia coli* K-12 carrying an Ent plasmid," Infection and Immunity 13:1005-1007 (1976).

Based on the Wilkins and the Falkow *et al.* studies, *E. coli* K-12 is nonpathogenic and nontoxigenic to cattle. K-12 is also not anticipated to lead to any changes in the endogenous gut flora of the animals as K-12 lacks the ability to colonize the animal gut.

3. Rabbits

E. coli K-12 was used as a non-pathogenic negative control in a study to determine the shared virulence properties between strains of *Hafnia alvei* isolated from diarrheal stools of children and enteropathogenic *E. coli*. In the negative control, 10^9 *E. coli* K-12 was inoculated into the 10 cm long intestinal loops of an adult New Zealand white rabbit that had fasted for 48 hours. After 20 hours, the rabbit was sacrificed and the inoculated intestinal loops were examined for fluid accumulation and other gross pathological changes. Histological sections of the ileal intestinal loop determined that the *E. coli* K-12 negative control lacked the ability to attach to the intestinal cells, which was observed to occur in the pathogenic *H. alvei* strains.[56] These results are consistent with the inability of *E. coli* K-12 to colonize the gut as described in Section I of this filing.

a) Other Animals

Scientific articles on the effects of *E. coli* K-12 on other animal species were not identified in an extensive search of the literature. It can be concluded that *E. coli* K-12 is nontoxigenic and nonpathogenic in other animals because *E. coli* K-12 does not express any toxins, is a common constituent in the gastro-intestinal tract of man and animals, is unable to colonize the gut, and the data in the cited human, chicken, rabbit, and cattle studies support its lack of toxicity and pathogenicity.

B. ANIMAL SAFETY ASSESSMENT FOR L-METHIONINE 85%

The L-methionine 85% was tested for acute oral toxicity in the rat according to OECD 423, in 2011 (**Attachment 8**). The results show no mortality or any other clinical signs during the course of the study, including no effects on body weight gain. Acute exposure to L-

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[56] Albert, M., et al, "Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*," Journal of Medical Microbiology 37(5):310-314 (1992).

methionine 85% administered orally to rats at 2000 mg/kg did not induce mortality or any signs of toxicity. The acute oral LD_{50} is > 2000 mg/kg bw.

The subchronic exposure study using rats summarized below (see Section V.C.) found no effects that differed from the well-known toxicological profile of methionine established in previous studies.[57] The primary and well-known toxicological effect of methionine is hemolytic anemia; this effect was also observed in the current study. Treatments at all doses induced no mortality and no significant clinical signs. The No Observed Adverse Effect Level (NOAEL) reported by the laboratory was 250 mg/kg bw/day, based on suggestive changes in hematological effects at 500 mg/kg bw/day. However, this selection of a NOAEL is very conservative because the effects reported at that dose are not significantly adverse. Very few measures of hematological changes were reported at 500 mg/kg bw/day and those that were reported were not sufficiently significant to be considered adverse. Furthermore, the results obtained in the subchronic feeding study of L-methionine 85% were very similar to those obtained by Toue, et al.[58] The administered doses in the Notifier's study were 0.4%, 0.7%, and 1.4% for female rats and 0.4%, 0.9% and 1.8% for male rats. Toue et al. reported similar small changes in hematological parameters at 1.2% in the diet of rats and frank changes at 2.4%. It is a matter of judgment whether the effects at 0.7-0.9% observed in the Notifier's study should be considered adverse, but the important point is that similar effects were observed in both studies at similar exposures. The Toue et al. study used pure crystalline methionine and the Notifier's study used L-methionine 85%. Similar inconsistent effects were observed in both studies at intakes of approximately 1% dietary methionine with more certain changes observed at exposures closer to 2% dietary methionine, indicating that the L-methionine 85% is substantially equivalent to pure methionine when fed in the diet of rats and does not introduce any new toxicological considerations or uncertainty.

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57 *See, e.g.*, Benevenga, N. and Steele, R., "Adverse effects of excessive consumption of amino acids," Annu. Rev. Nutr. 4: 157-181 (1984); Harter, J. and Baker, D., "Factors affecting methionine toxicity and its alleviation in the chick," J. Nutr., Vol. 108(7): 1061-1070 (1978); Mengel, C. and Klavins, J., "Development of hemolytic anemia in rats fed methionine," J. Nutr., 92(1): 104-110 (1967).

58 Toue, S., et al. "Screening of Toxicity Biomarkers for Methionine Excess in Rats," J. Nutr., 136, 1716S–1721S (2006).

We conclude that L-methionine 85% is substantially equivalent to DL-methionine for the purpose of nutrient supplementation in the target animals. As discussed above, the material balance determined by analysis (see Attachment 2) indicated that no components of L-methionine 85% differ significantly from normal dietary constituents of livestock and domestic animals. Consequently, supplementation of the diet with L-methionine 85% will not expose the animals to any substance not already in the diet. Substantial equivalence of the L-methionine 85% to DL-methionine for the purpose of dietary supplementation confirms that the product does not present a reasonable risk of harm to any animal when fed at levels consistent with Good Feeding Practice.

C. ANIMAL SAFETY ASSESSMENT FOR RESIDUALS

As explained below, we conclude that the manufacturing process for L-methionine 85% does not generate residual contaminants at levels that might present a risk of harm to either animals consuming the supplement directly or to humans. This conclusion is based, first, on the common occurrence of all residuals in the conventional diets of livestock, domestic animals, and humans as commonly occurring metabolites of living organisms or as products of food processing and handling. Second, the very small parts per million (ppm, mg/kg) amounts of every residual contributing to the compositional material balance will be consumed in insignificant amounts by any animal consuming the supplement.

The purification process for methionine through (b) (4) (Section III.C. above) and the subsequent (b) (4) steps lead to insignificant levels of impurities in the final product. A residual analysis of the final L-methionine 85% is provided in Attachment 2. The final product contains at least the minimum 85% methionine and an average percentage of about 2.3% of other amino acid types. As amino acids serve as an essential nutrient in the animal diet, the consumption of methionine and the other low levels of amino acids by animals is safe.[59]

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[59] Rose, W., "The Nutritive Significance of the Amino Acids," Physiol. Rev. 18(1): 109-136 (1938), available at http://physrev.physiology.org/content/18/1/109.full.pdf (last accessed July 31, 2012); *see also* 21 C.F.R. § 172.320.

The other components, comprising approximately (b) (4) of the final product, are all, with the exception of (b) (4) 1.65%, well below 1% of the final product. The residuals present are mostly normal cell metabolites and none of the compounds are classified as known carcinogens. Several of the other components, such as cystathionine, are precursors in the methionine production pathway, and most likely are present in the final product due to overexpression of the methionine pathway.[60] Other compounds, such as spermidine, are normal cell metabolites normally found in both *E. coli* and animal cells. Compounds such as putrescine and cadaverine are normal breakdown products due to the (b) (4) . Table 11 contains all of the components that were detectable in the final product, with the exception of compound groups such as amino acids, cations, and organic acids, which have been removed because these are easily identifiable as normal cell metabolites that do not pose any risk, especially at these very low concentrations.

In addition, the impurities in L-methionine 85% identified in Attachment 2 are common metabolites found in all food and food additive products produced by bacterial fermentation. As a result, these impurities are already consumed by man and animals when consuming a food or food additive produced through fermentation of a bacterial cell. Therefore, no risk is expected from these substances.

Table 10 below displays the results of calculations of concentrations of residuals, based on inclusion of L-methionine 85% up to 0.3% in finished animal feed. As described previously, the level of supplementation depends on a number of variables, including the target species, the animal's age, and its current diet, and is determined on a case-by-case basis according to good feeding practice. Therefore, for purposes of the risk assessment for the potential impurities present in the L-methionine 85%, we have chosen 0.3% as an example supplementation rate. On this basis, only the impurities present at 0.1% or higher are included in Table 10. As shown in this table, the impurities will account for an insignificant amount of the animal's total diet. For

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[60] Mondal, S., Das, Y., and Chatterjee, S., "Methionine production by microorganisms," Folia Microbiol (Praha). 41(6):465-472 (1996).

example, (b) (4) which is the highest impurity in the product and is present at 0.73%, will only account for 0.00219% of the total diet, which translates to about 21.9 mg per 1 kg of animal feed. Most of the other impurities are present at levels less than half of this amount. Therefore, the residual impurities present in L-methionine 85% will be an insignificant portion of an animal's diet.

For the typical use of the supplement at 0.3% in the diet of a young pig weighing 60 kg and consuming approximately 2.4 kg of feed per-day[61] (assuming feed comprises the complete diet), the exposure to (b) (4) at 22 mg/kg of feed will be approximately 53 mg/day, or less than 1 mg/kg of body weight. (b) (4) is a minor, but common metabolite of (b) (4) in living organisms for which there is no apparent potential for adverse effects at such a low exposure.[62] Similarly, a small chicken weighing 800 grams and consuming 140 grams of feed per day (again, assuming the feed comprises 100% of the diet)[63] supplemented with 0.5% L-methionine 85%[64] containing (b) (4) (37 mg/kg feed) will be exposed to approximately 5 mg/day, or about 6 mg/kg of body weight per day. Exposures to all other residual metabolites will be smaller than these examples. The occurrence of all of these residual impurities as metabolites in virtually all living organisms and their general occurrence in the diet, even if in very small amounts, indicates that these very small exposures do not present a potential risk of harm to either the animals consuming L-methionine 85% or humans consuming animal tissues or products derived from the animals consuming the supplement.

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61 SAX'S Dangerous Properties of Industrial Materials, 11th Edition. Table 2. 0.3% is a typical level for methionine supplementation in the diet of pigs, NAS/NRC (2012). Nutrient Requirements of Swine. National Academy of Sciences, National Research Council, Board on Agriculture and Natural Resources, p. 211.

(b) (4)

63 *Id.*

64 0.25% is a typical or average level for methionine supplementation in the diet of chickens, *see* National Academy of Sciences/National Research Council, "Nutrient Requirements of Poultry," p. 81 (1994); Fanatico, A., "Organic Poultry Production: Providing Adequate Methionine," ATTRA, Table 1, p. 3 (2010), available at http://www.slideshare.net/ElisaMendelsohn/organic-poultry-production-providing-adequate-methionine.

Table 10: Animal exposure percentage to impurities from consumption of L-methionine 85% produced from *E. coli*

Compound	Percent in L-Methionine 85%*	Percent in Feed (%)**	Amount in Feed (mg/kg feed)**

(b) (4)

* The calculated average value from three lots of the L-methionine 85% (Attachment 2)
** These values were calculated based on 0.3% L-methionine 85% supplementation for a young pig.

Table 11: Residual analysis of final product

Compound	Description	Method
(b) (4)	Normal cell metabolite	Liquid chromatography and UV detection
	Normal cell metabolite; amino acid isomer	Gas chromatography - flame ionization detection after derivatisation with silylated reagent
	Normal cell metabolite; Intermediate in amino acid cysteine synthesis in *E. coli*	Gas chromatography - flame ionization detection after derivatisation with silylated reagent
	Normal cell metabolite due to oxidation of methionine	Gas chromatography - flame ionization detection after derivatisation with silylated reagent
	Normal cell metabolite; precursor to methionine biosynthesis	Gas chromatography – Mass spectrometry detection after derivatisation with silylated reagent
	Normal cell metabolite due to degradation of methionine or cystathionine	Gas chromatography – Mass spectrometry detection after derivatisation with silylated reagent
	Normal cell metabolite	Liquid chromatography and UV detection after derivatisation with ninhydrine
		(b) (4)
	Normal cell metabolite; produced during degradation of the amino acid L-cysteine	Anion exchange liquid chromatography and conductimetric detection
	Amino acid breakdown product due to hydrolysis	Cation exchange liquid chromatography and conductimetric detection
	Normal cell metabolite; produced from amino acid arginine	Cation exchange liquid chromatography and conductimetric detection
	Normal cell metabolite	Cation exchange liquid chromatography and conductimetric detection

V. HUMAN CONSUMPTION AND SAFETY

The L-methionine 85% is intended for use as a nutrient for animal consumption. Ordinarily, a GRAS notice will address the potential human dietary consumption of a component of animal feed due to consumption of animal products and tissues in which the component may be present. In this case, however, there is no need to determine the estimated daily intake (EDI) of the L-methionine 85% for human consumption. The L-methionine 85% and any of the

residual impurities described above (which includes low levels of other amino acids) will be metabolized when the animal consumes and digests its food (like other amino acids). Consequently, the L-methionine derived from the modified *E. coli* K-12 will be indistinguishable from methionine derived from other sources.

In this regard, the European Food Safety Authority's (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has recently reviewed the safety and efficacy of methionine, methionine sodium salt, the hydroxy analogue of methionine, and the calcium salt of methionine hydroxy analogue for use in the diets of all animal species.[65] In the report the EFSA Panel noted that methionine and methionine-based additives in the feed of animals result in the incorporation of all absorbed methionine in tissue protein. Doses exceeding the methionine requirement of the animal will be excreted. Consequently, no free methionine occurs or accumulates in target animal tissues, and the only form of methionine that humans will be exposed to from its use in animal feed is in the form of protein that will be digested, absorbed, and metabolized consistent with human nutrient needs. The absence of residual methionine in the tissues of animals consuming any form of methionine in its diet will, therefore, not result in a subsequent human exposure to the additive or pose a safety issue. As indicated by the analytical values displayed in Attachment 2 and Table 10, residual components of L-methionine 85% are at levels so low as to present no risk of harm in humans consuming the tissues of food animals fed the nutrient. All residual constituents are common metabolites or metabolic products, and will be either excreted or metabolized. Therefore, they present no exposure risk to humans consuming tissues or products from the target animal. A review of the publicly available literature does not reveal information demonstrating that any of these residual constituents appears to present a risk of accumulation or harm to humans at the levels that would be consumed from animal tissue.[66] It should also be noted that L-methionine is an essential amino

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65 *See* EFSA (2012), footnote 8.

66 The databases reviewed are PubMed, Google Scholar, National Toxicology Program, ToxNet, International Programme on Chemical Safety, and the Environmental Protection Agency's Integrated Risk Information System. Literature searches were conducted for (b) (4). Several substances were not detected in the residue testing (cadaverine, spermidine, homocysteine, nickel, chromium, mercury, and arsenic) and

(footnote continued)

acid for humans, naturally present in a number of foods, and is approved for direct addition to human food in 21 C.F.R. § 172.320 as an amino acid.

The final production strain of modified *E. coli* K-12 does not contain any antibiotic resistance markers. Although (b) (4)

Nevertheless, to support the safety of any potential human dietary exposure to L-methionine 85%, Notifier has conducted *in vivo* genotoxicity studies, a subchronic oral toxicity study, an acute oral toxicity study, and a developmental toxicity study. The studies are summarized below. In all cases, the L-methionine 85% was the substance tested.

A. ACUTE ORAL TOXICITY

Acute oral toxicity studies were conducted using L-methionine 85% in the mouse and the rat. The test material was administered to female rats as a single gavage dose at a level of 2000 mg/kg of body weight. No mortality or clinical signs were observed during the studies. The estimated oral LD_{50} value for female rats was determined to be greater than 2000 mg/kg. The study reports are included as **Attachment 8**.

Study:	**L-Methionine Lab 4179 Acute Oral Toxicity Study in the Mouse: Acute Toxic Class Method (OECD) 423) (Study No. 20100288TRP)**
GLP:	Yes
Animals:	Female SPF Swiss – Crl: OF1 strain mice, three per step
Animal Husbandry:	After receipt, animals were acclimated for a period of at least 5 days. During acclimation and throughout the study, the animals were individually housed. The room in which the test animals were housed was

so were not evaluated for safety. The safety of the remaining impurities, such as (b) (4), were considered to be sufficiently well established that a literature search was unnecessary.

maintained at a temperature ranging from 20-24°C, a relative humidity of 45-65%, and a 12-hour light/12-hour dark cycle. Where variations of these conditions occurred, these were not considered to have an adverse effect on the study outcome.

Test Material: L-methionine 85%

Dosing Method: The test substance was administered as a single gavage dose at a volume of 10 ml/kg of body weight.

Dose Levels: The test compound was administered at a single dose level of 2000 mg/kg body weight.

Observations: Clinical observations and mortality checks were conducted at 30 minutes and 3-4 hours after test material administration and daily thereafter for 14 days. Body weights were determined on the day of test material administration (Day 1), at Days 2, 5, 8, 11, 14, and at Day 15 (at termination of the in-life phase).

Mortality. No mortality was observed during the study.

Body weights. All test animals exhibited body weight gain throughout the study.

Clinical signs. No clinical signs were noted during the study.

Macroscopic findings. No macroscopic observations were seen at necropsy.

Conclusion: The estimated oral LD_{50} values for female rats was determined to be greater than 2000 mg/kg of body weight.

Study: **L-Methionine Lab 4179 Acute Oral Toxicity Study in the Rat: Acute Toxic Class Method (OECD) 423) (Study No. 20100289TRP)**

GLP: Yes

Animals: Female SPF Sprague-Dawley – Crl: OFA strain rats, three per step, six in total

Animal Husbandry: After receipt, animals were acclimated for a period of at least 5 days. During acclimation and throughout the study, the animals were housed in groups of 5. The room in which the test animals were housed was

maintained at a temperature ranging from 20-24°C, a relative humidity of 45-65%, and a 12-hour light/12-hour dark cycle. Where variations of these conditions occurred, these were not considered to have an adverse effect on the study outcome.

Test Material: L-methionine 85%

Dosing Method: The test substance was administered as a single gavage dose at a volume of 10 ml/kg of body weight.

Dose Levels: The test compound was administered at a single dose level of 2000 mg/kg body weight.

Observations: Clinical observations were conducted prior to dosing. Clinical observations and mortality checks were conducted at 30 minutes and 3-4 hours after test material administration and daily thereafter for 14 days. Body weights were determined on the day of test material administration (Day 1), at Days 2, 5, 8, 11, 14, and at Day 15 (at termination of the in-life phase).

Mortality. No mortality was observed during the study.

Body weights. All test animals exhibited body weight gain throughout the study.

Clinical signs. No clinical signs were noted during the study.

Macroscopic findings. No macroscopic observations were seen at necropsy.

Conclusion: The estimated oral LD_{50} values for female rats was determined to be greater than 2000 mg/kg of body weight.

B. GENOTOXICITY TESTING

An *in vivo* mammalian erythrocytes micronucleus test was conducted in rat bone marrow treated with L-methionine 85% at 50, 100, and 200 mg/mL, together with vehicle and positive controls. In the mouse micronucleus test, animals are treated with the test substance and the frequency of micronucleated cells (cells having an unusually small nucleus) is determined at a specified time after treatment. If an exposed group of animals shows significantly higher frequencies of micronucleated cells than do the untreated control animals, the test substance is

considered to be capable of inducing structural and/or numerical chromosomal damage. It is also conventional in the test to score polychromatic erythrocytes (red blood cells) (PCEs) and normochromatic erythrocytes (NCEs). PCEs are immature erythrocytes and are scored per animal. The percentage of PCEs among the total erythrocyte population in the bone marrow is scored for each dose group as an indicator of chemical-induced toxicity. The absence of a change in the normal ratio of PCEs to NCEs indicates the absence of toxicity. The L-methionine 85% test material did not induce any statistically significant increases in the in the PCE:NCE ratio. On this basis, the test material was shown not to be genotoxic within the limitations of the study protocol. The study is included as **Attachment 9.**

In combination with the *in vivo* mammalian erythrocytes micronucleus test, a Comet assay was conducted at the same dose levels, with vehicle and positive controls. No statistically significant increase in the median percentage of tail DNA at the two analyzed doses of 2000 and 1000 mg/kg/day was seen as compared to the negative control. Furthermore, no statistically significant increase in the occurrence of ghost cells was noted at any of the doses analyzed. The study is included with Attachment 9.

A separate *in vivo* Comet Assay in the rat was also conducted. The dose levels were 500, 1000, and 2000 mg/kg, and included vehicle and positive controls. Statistically significant decreases in the number of ghost cells were noted at both analyzed doses of 2000 and 1000 mg/kg, but this decrease was considered to have no meaning in terms of cytotoxicity. No statistically significant increase in the percentage of DNA in tail median was observed at the two highest doses tested of 2000 and 1000 mg/kg/day, and the L-methionine 85% was considered not to be genotoxic under the conditions of the study. The study is included as **Attachment 10.**

Study: ***In Vivo* Mammalian Erythrocytes Micronucleus Test Performed in Rat Bone Marrow Combined to The Comet Assay in the Liver (Study No. FSR-IPL 110501)**

GLP: Yes

Animals: Micronucleus test: Male and female OFA Sprague Dawley rats
Comet assay: Male OFA Sprague Dawley rats

Test Material: L-methionine 85%. For the preliminary and confirmatory assays, a suspension at a maximum concentration of 200 mg/mL was used, while in the main genotoxicity assay, three suspensions at concentrations of 200, 100, and 50 mg/mL were used.

Dose Level: Doses were administered at 10 mL/kg. For the preliminary and confirmatory assays, the dose level was 2000 mg/kg, while in the main genotoxicity assay, the dose levels were 2000, 1000, and 500 mg/kg.

Group	Treatment	Dosage	# Animals Micronucleus		# Animals Comet
			M	F	M
1	Vehicle^	--	5	5	4
2	Test*	2000 mg/kg	5	5	4
3	Test	1000 mg/kg	5	5	4
4	Test	500 mg/kg	5	5	4
5	Cyclophosphamide	25 mg/kg	5	5	--
6^	Dimethylhydrazine	10 mg/kg	--	--	3

^ Male rats were treated with the test item and with the vehicle for both the micronucleus test and the comet assay.
* For the group treated with the high dose in the micronucleus assay, 2 supplementary animals of each sex were treated in parallel to the 5 others, for a total of 7.

Controls: Vehicle and positive controls were used in parallel with the test material. The positive control for the micronucleus test was 25 mg/kg of cyclophosphamide, administered intraperitoneally under a volume of 10 mL/kg. The positive control for the comet assay was 10 mg/kg dimethylhydrazine, administered orally under a volume of 10 mL/kg.

Exposure: Preliminary and Confirmatory Test

A preliminary assay was conducted in groups of 2 male and 2 female rats, while a confirmatory assay was conducted to confirm the maximum non-lethal dose using a group of 5 males and 5 females. Animals were treated twice with 2000 mg/kg/day. In both assays, no clinical signs were observed after 24 hours after the second treatment. Two lesser doses of 1000 and 5000 mg/kg/day also were tested. Consequently, 2000 mg/kg/day was the highest dose selected for the main study.

Main Micronucleus Test/Comet Assay

In the main study, treatment took the form of 3 successive administrations at 24-hour intervals by oral route. Samples were taken at 3-6 hours after the last treatment. The positive control for the micronucleus test was administered as a single injection 24 hours before sampling. The positive control for the comet assay was administered 3-6 hours before sampling. 4 out of 5 males per group were assigned for cell isolation and assessment of DNA fragmentation.

Results:

Micronucleus test

The mean number of micronucleated PCE observed in the negative control animals was within the range of historical values. Statistically significant increases in the frequency of micronucleated PCE were noted in the positive control group, demonstrating the sensitivity of the animal strain to a clastogenic agent. No statistically significant decrease in the PCE:NCE ratio was noted in the 3 test treatment groups when compared to the negative control group.

Comet Assay

Cell viability in the control group was superior to 50%, while the doses of 2000 and 1000 mg/kg/day gave relative cell viability using the Trypan blue vital dye exclusion technique superior to 70% and were analyzed. Toxicity of the test item was evaluated by means of the non-denaturing fast halo assay. No apoptotic cells were noted. Furthermore, no statistically significant increase in the number of necrotic cells was noted at the doses tested of 2000 and 1000 mg/kg/day when compared to the negative control. A statistically significant decrease in the number of necrotic cells was noted at the 2000 mg/kg dose with a value of 4.5% vs. 7.9% in the vehicle control group. This decrease was considered to have no meaning in terms of cytotoxicity and/or biological activity.

In the positive control group, the median percentage of DNA in tail per slide was statistically increased compared to the control group. The medians percentages of DNA in tail in vehicle and in positive control groups were consistent with historical values. No statistically significant increase in the median percentage of tail DNA at the two analyzed doses of 2000 and 1000 mg/kg/day was seen as compared to the negative control. Furthermore, no statistically significant increase in the occurrence of ghost cells was noted at any of the doses analyzed.

Conclusion: The test article was considered to be non-genotoxic under the conditions of the study. Systemic exposure was proven by checking the concentration of L-methionine 85% in rat plasma samples.

Study: ***In Vivo* Comet Assay in the Rat Performed on Stomach (two treatments, one sampling time) (Study No. FSR-IPL 110901)**

GLP: Yes

Animals: Male OFA Sprague Dawley rats, 5 (treated and negative control) and 4 (positive control) per group, although 4 and 3, respectively, were analyzed.

Test Material: L-methionine 85% in suspension.

Dose Level: Doses were administered under a volume of 10 mL/kg, at concentrations of 2000, 1000, and 500 mg/kg.

Controls: Vehicle and positive controls were used in parallel with the test material. The positive control was methylmitronitrosoguanidine, administered orally as a single dose at 20 mg/kg.

Exposure: Treatment took the form of 2 administrations at 24-hour intervals by oral route. Samples were taken at 3-6 hours after the second treatment. The positive control was administered 3-6 hours before sampling.

Results: The 2000, 1000 and 500 mg/kg doses gave acceptable relative cell viability. No apoptotic cells were noted in the halo assay. In return, statistically significant increases in the number of necrotic cells were noted at the 2 analyzed doses of 2000 and 1000 mg/kg when compared to the negative control. [The test item thus presents a toxic activity toward stomach cells, with a dose-response effect.] Statistically significant decreases in the number of ghost cells were noted at both analyzed doses of 2000 and 1000 mg/kg, but this decrease was considered to have no meaning in terms of cytotoxicity. No statistically significant increase in the percentage of DNA in tail median was observed at the 2 highest doses tested of 2000 and 1000 mg/kg/day.

Conclusion: The test article was considered to be non-genotoxic under the conditions of the study.

C. SUBCHRONIC ORAL TOXICITY (PRELIMINARY AND MAIN STUDIES)

A 13-week subchronic oral toxicity study in the rat was conducted, with an additional four-week withdrawal period. A preliminary study utilized dose levels of 500, 1000, and 2000 mg/kg/bw, with a vehicle control. A decrease in body weight gain and food consumption was noted at the 2000 mg/kg/bw dose, so the dose levels in the main study were 250, 500, and 1000 mg/kg/bw. Treatments at all doses induced no mortality and no clinical signs. Treatment at 1000 mg/kg/bw could induce a haemolytic anaemia, and at 500 mg/kg/bw, haematologic changes were noted. Treatment at 1000 mg/kg/bw and 500 mg/kg/bw induced adrenal cortical zona fasciculate vacuolation only in males, but may not be associated as a toxic response. Therefore, the No-Observed Adverse Effect Level (NOAEL) was set at 250 mg/kg/bw. These studies are included as **Attachment 11.**

Study: **L-Methionine Lab 4179 13-Week Oral Toxicity Study in the Rat Including Recovery and Toxicokinetics (Study No. 20100291TRPB)**

GLP: Yes

Animals: Male and female SPF Sprague-Dawley – Crl: OFA strain rats.

Test Material: L-methionine 85%

Dose Levels: A preliminary test was conducted on four groups of animals with each having 5 males and 5 females. These animals were dosed once daily for 14 days at control, 500, 1000, and 2000 mg/kg/bw. A decrease in body weight gain and food consumption was noted at the 2000 mg/kg/bw dose, so the dose levels in the main study were 250, 500, and 1000 mg/kg/bw. Satellite groups were used only for toxicokinetic assessment. Withdrawal groups were included to investigate delayed occurrence, persistence, or reversibility of findings. These animals went through treatment, then had a four week, treatment-free recovery period.

Group	Treatment	Dosage	# Animals Preliminary		# Animals Main		# Animals Satellite		# Animals Withdrawal	
			M	F	M	F	M	F	M	F
1	Vehicle	--	5	5	10	10	3	3	10	10
2	Test	1000 mg/kg	5	5	10	10	6	6	10	10
3	Test	500 mg/kg	5	5	10	10	6	6	--	--
4	Test	250 mg/kg	5	5	10	10	6	6	--	--

Controls: A vehicle control was used in parallel with the test material.

Exposure: Treatment took the form of once daily administrations by the oral route for 13 consecutive weeks. At the end of the treatment period, animals from the treatment withdrawal group were kept for four additional weeks without treatment.

Observations: General observations were taken before the first dose and daily thereafter, at ~1 hour post-treatment. Full clinical examination was conducted weekly, at ~1 hour post-treatment. Observations were conducted prior to dosing. Functional and neurobehavioral tests were conducted prior to first dose, on the 6th week, and during the last week, using animals from the main and treatment withdrawal groups, only. Body weights were determined on the day of randomization, day before test material administration (Day 1), every 7 days during the study (Days 7, 14, 21, etc.),and on the day of necropsy.

Results:

Preliminary test

Mortality. No mortality was observed during the study.

Body weights. A decrease in body weight gain and food consumption was noted at the 2000 mg/kg/bw dose.

Clinical signs. No clinical signs were noted during the study.

Main Study

Mortality. Three males (two from the control group and one treated at 1000 mg/kg/bw) were found dead on D5, D42, and D85, and two females (one from the satellite control group and one from the main group treated at 1000 mg/kg/bw) were found dead on D56 and D86.

Body weights and Feed Consumption. A statistically significant lower body weight gain was noted from D42 to D91 in males at 250 mg/kg/bw and from D14 to the end of treatment withdrawal period in males at 1000 mg/kg/bw when compared with the control group. No difference was noted in males treated at 500 mg/kg/bw and in females at all any dose level.

A slightly lower food consumption was noted in males treated at 250 mg/kg/bw and 1000 mg/kg/bw in comparison with the control group. A statistically lower food consumption was noted on week 4 in males treated at 1000 mg/kg/bw. A lower food consumption (-17% in comparison with control group) was noted on week 5 in males treated at 1000 mg/kg/bw due to two unexpectedly high food consumption in cages

3 and 4 of the control group. A statistically significant higher food consumption was noted on week 4 and 6 in females treated at 1000 mg/kg/bw (respectively +9% and +14%).

Clinical signs. No clinical signs were noted during the study. Isolated clinical changes were noted such as chromodacryorrhea (from D77 to D80, in one or two males at 1000 mg/kg/bw), ptosis, increasing grooming (on D35 in one female at 500 mg/kg/bw), recumbent position and absence of spontaneous locomotor activity (on D92 in one female control) or alopecia of the foreleg (from D53 to D90 in one female at 1000 mg/kg/bw).

Macroscopic findings. No relevant observation was noted at necropsy. Isolated observations were noted such as punctate change in liver, dark area in spleen in control or treated animals. As three animals (one male and one female of the control group and one male treated at 500 mg/kg/bw), died just before necropsy on D92, exsanguination was not total, leading to a dark coloration of some organs. In animals that died during the study, there was mainly a dark coloration of some organs leading to the non exsanguination and lung with swollen appearance and mottled.

Conclusion: Treatments of 250, 500 and 1000 mg/kg/bw administered daily by the oral route for 13 weeks in rats induced no mortality and no clinical signs. Treatment at 1000 mg/kg/bw could induce a hemolytic anemia. At 500 mg/kg/bw, hematologic changes were noted (lower red blood cell count and higher total bilirubin) and could suggest the beginning of hemolytic anemia. In previous studies, it was established that rats fed excessive amounts of methionine developed a moderate degree of anemia. This is the effect seen with sulfur amino acids.[67] Treatment at 1000 mg/kg/bw and 500 mg/kg/bw induced adrenal cortical zona fasciculate vacuolation only in male. The degree of adrenal cortical vacuolation was akin to that seen in metabolic disturbance and is not considered a toxic response.

Therefore, the No-Observed Adverse Effect Level (NOAEL) corresponds to 250 mg/kg/bw.

D. DEVELOPMENTAL TOXICITY

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67 Klavins, J. and Mengel, C., "Development of hemolytic anemia in rats fed methionine," J. Nutrition 92 (1967).

Preliminary and full studies were conducted to measure the effects of L-methionine 85% on embryo-fetal development when administered by the oral route. Dose levels in both studies were 125, 250 and 500 mg/kg. In the preliminary study, all treatments did not induce mortality or systemic toxicity in pregnant female Sprague Dawley rats. No external abnormalities in fetuses were reported. In the full study, no mortality or clinical signs were seen. No treatment levels gave rise to any morphological changes that were considered to be related to maternal treatment. As a consequence of a treatment-related reduction in fetal weights at 500 mg/kg/bw fetal ossification was slightly retarded. The NOAEL for embryo-fetal development was considered to be 250 mg/kg/bw, based on a small reduction in average fetal weights. This endpoint is not a frank toxic effect and should have no adverse implication with regard to the use of methionine in livestock or domestic animals. These studies are included as **Attachment 12.**

Study:	**Preliminary Study for Effects on Embryo-Foetal Development in the Rat by the Oral Route (Study No. 20100292STP)**
GLP:	No
Animals:	28 mated female OFA Sprague Dawley rats, in four groups of seven rats.
Test Material:	L-methionine 85%.
Dose Level:	Doses were administered under a volume of 10 mL/kg, at concentrations of 125, 250 and 500 mg/kg, with one group receiving the vehicle.
Exposure:	Treatment took the form of once daily administrations by the oral route from Day 6 (implantation) to Day 19 (one day before termination).
Observations:	Morbidity/mortality checks were performed twice daily from Day 6 onward. Clinical observations were performed before the first dosing and then daily thereafter. Body weight was recorded on Day 1, Day 5, and from Day 6 to Day 20. On Day 20 of pregnancy, all mated females were necropsied and all fetuses were examined.
Results:	*Mortality*. No mortality was seen throughout the study. *Body weights*. No difference in body weight gain was noted between the treated and control groups. *Clinical signs*. No clinical signs were noted during the study.

Necroscopic Examination – Mated Females
Macroscopic examination: No relevant observation was noted at necropsy.
Uterus weight: No difference in uterus weight was noted between control and treated females.
Litter data: There were no changes in the number of corpora lutea, in the number of live fetuses and early resorptions between the treated and the control group. The number of implantation sites was slightly lower in treated females at 500 mg/kg due to 2 females which had only 3 or 2 implantation sites.

Necroscopic Examination – Fetuses
Macroscopic examination: No fetus was found dead following the caesarian section of females dosed with the vehicle or with the test sample, at any dose. Except for dark point/area seen at the same incidence in all groups and one fetus in the intermediate dose-group with omphalocele, no observation was noted at necropsy.
Fetus weight: No differences were seen in fetal weights between the treated and the control groups.

Conclusion: Under the conditions of the study, treatments at 125, 250 and 500 mg/kg by the oral route did not induce mortality or systemic toxicity in pregnant female Sprague Dawley rats. No external abnormalities in fetuses were reported. Based on these results, the doses for the main study were set at 125, 250 and 500 mg/kg by the oral route.

Study: **Study for Effects on Embryo-Foetal Development in the Rat by the Oral Route (Study No. 20100293TRPB)**

GLP: Yes

Animals: 92 mated female OFA Sprague Dawley rats, in four groups of 10 rats, plus four satellite groups of three.

Test Material: L-methionine 85%.

Dose Level: Doses were administered under a volume of 10 mL/kg, at concentrations of 125, 250 and 500 mg/kg, with one group receiving the vehicle.

Exposure: Treatment took the form of once daily administrations by the oral route from Day 6 (implantation) to Day 19 (one day before termination).

Observations: Morbidity/mortality checks were performed twice daily from Day 6 to Day 20. Clinical observations were performed before the first dosing and then daily thereafter. Body weight was recorded on Day 2, Day 4; and

Day 5, and from Day 6 to Day 20. Blood for toxicokinetic assessment was collected from satellite groups on Day 6 and Day 19. On Day 20 of pregnancy, all mated females were necropsied and all fetuses were examined.

Results:

Mortality. No mortality was seen throughout the study. Signs of abortion (blood near genital orifice and in cage) were seen in one female on Day 15.

Body weights. No difference in body weight gain was noted between the treated and control groups.

Clinical signs. No clinical signs were noted during the study.

Necroscopic Examination – Mated Females
Following signs of abortion, one female was euthanized on Day 15. A lot of corpora lutea on the ovaries were noted for this female.
Macroscopic examination: No relevant observation was noted at necropsy.
Uterus weight: No difference in uterus weight was noted between control and treated females. The uterus weight was slightly lower in females treated at 500 mg/kg/bw due to one female which had only three live fetuses.
Pre-implantation loss, number of live foetuses, post-implantation loss, fetal incidence and litter data: There were no relevant changes in the number of corpora lutea, in the number of live and abnormal fetuses, in the number of normal and abnormal dead fetuses and in the early and late resorptions. A statistically significant lower implantation site number was noted in animals treated at 250 and 500 mg/kg/bw when compared to the control group.

Necroscopic Examination – Fetuses
Macroscopic examination: In the treatment group at 500 mg/kg/bw, one fetus showed a decreased size of limbs, a swollen and dark aspect of the abdomen and a missing anus and tail. Except for these observations of this fetus and for dark point/area or size of placenta seen at the same incidence at any dose, no relevant observation was noted at necropsy.
Fetus parameters: A statistically significant lower fetus weight was noted in all treated groups when compared to control group (-5% in lowest dose group, -3% in intermediate and -9% in the highest dose group). The fetuses were slightly smaller (-4% and -3% respectively) in the 250 mg/kg/bw and 500 mg/kg/bw treated groups when compared to the control group with respect to the caudo-cranial measurement. A statistically significant lower placenta weight was also noted in the 250 and 500 mg/kg/bw treated groups.

Fetal pathology: There was considerable inter-litter and intra-litter variation in fetal size and fetal weight and this was mirrored by the degree of variation in ossification between individual fetuses and litters. Fetuses in some litters were large and well ossified, whereas other fetuses were small and poorly ossified. Ossification of fetuses in groups 2 and 4 (125 and 500 mg/kg/bw tended to be slightly less than in the control group and group 3 (250 mg/kg/bw), which reflected the slightly lower group mean body weights in groups 2 and 4. Examination of the skeletal data on an individual basis showed that the reduction in ossification appeared to be closely associated with fetuses of lower body weight. As a consequence of a treatment-related reduction in fetal weights at 500 mg/kg/bw fetal ossification was slightly retarded.

Conclusion: Under the conditions of the study, treatments at 125, 250 and 500 mg/kg by the oral route did not give rise to any morphological changes that were considered to be related to maternal treatment. As a consequence of a treatment-related reduction in fetal weights at 500 mg/kg/bw fetal ossification was slightly retarded. The NOAEL for embryo-fetal development was considered to be 250 mg/kg/bw.

VI. CONCLUSION

Based on the documentation provided in this GRAS Notification, and as discussed above, Keller and Heckman LLP and our client Roquette Freres, have concluded that L-methionine 85% produced by a genetically modified *Escherichia coli* K-12 is GRAS via scientific procedures for use as a nutrient for animal consumption.

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TAB

1

HOW FDA APPROVED CHYMOSIN: A CASE HISTORY

by Eric L. Flamm

The U.S. Food and Drug Administration (FDA) has accumulated substantial experience with biopharmaceuticals, and now it has some with engineered food ingredients as well: it formally approved the first food ingredient made via recombinant DNA technology just over a year ago (*55 Fed. Reg.* 10932, March 23, 1990). The product is a chymosin enzyme (rennin) preparation derived from *Escherichia coli* K-12 carrying the bovine prochymosin gene. This article details the actual approval process used by FDA.

Chymosin

Chymosin (rennin) is the principal milk-clotting enzyme present in rennet[1,2]. It is a protease that hydrolyzes one bond in the kappa-casein protein of milk, cleaving it into two peptides. Kappa-casein normally stabilizes micelles in milk, but when it is cleaved, the micelles precipitate into curds. After the liquid whey is removed, the curds may be processed into cheese or other dairy products.

Traditionally, most commercial cheeses and other protease-dependent dairy products have been made using rennet isolated from the fourth stomachs of unweaned calves[2]. As consumption of veal fluctuates, so does the availability and price of calf rennet. Rennet was affirmed as GRAS (generally recognized as safe; see box for a detailed explanation of this term) by FDA in 1983 (*48 Fed. Reg.* 51151, Nov. 7, 1983).

Several forms of chymosin are found in calf rennet. The two most predominant and active are chymosin A and chymosin B, isozymes differing by a single amino acid[1,3]. (For the purposes of this discussion, however, they will not be distinguished.)

Calf chymosin is the final expression product of the prochymosin gene, which consists of 9 exons and 8 introns[4]. The primary translation product is inactive due to the presence of a 42 amino acid fragment at its amino terminal end[5]. Acid conditions in the calf stomach and during commercial processing cleave off this fragment, yielding active enzyme[1].

The prochymosin gene has been cloned into several microorganisms, where it produces prochymosin that can be acid-hydrolyzed into active chymosin[6-12]. Researchers have used a variety of techniques to demonstrate that the recombinant chymosin in all cases is functionally equivalent to rennet.

The First Chymosin Petition

FDA has received three petitions for chymosin derived from microorganisms, starting in 1987. The first (GRASP 8G0337) requested GRAS affirmation for a chymosin preparation derived from *Escherichia coli* K-12. After reviewing information in the petition and in the published literature, FDA concluded that the principal active component of the chymosin preparation is the same as that of rennet, and that the impurities of the chymosin preparation, which differ from the impurities of rennet, do not render the substance unsafe for its intended use. FDA therefore affirmed this preparation as GRAS for use as a replacement for rennet.

Establishing Identity

One FDA concern was to determine whether the cloned chymosin is identical to the chymosin in rennet. The petitioners provided published scientific data documenting that the prochymosin gene had been cloned and that it is properly expressed in its microbial hosts to make functional chymosin[6-12].

The petitioners used three lines of evidence to show that the correct gene had been cloned. The cloned DNA was digested with restriction enzymes and the resulting fragments were found to be the sizes predicted by the DNA sequence of the prochymosin gene[7-11]. The cloned DNA, and RNA synthesized from it, hybridized appropriately with the calf prochymosin gene[7-11]. Finally, the sequence of the cloned DNA corresponded to the amino acid sequence of the prochymosin protein[6,7].

The cloned prochymosin gene produced chymosin of the expected size and biological activity. Cloned chymosin has the same molecular weight as chymosin derived from calf rennet, as demonstrated by SDS polyacrylamide electrophoresis[8-14]. Cloned chymosin also has the same functional activity as chymosin derived from calf rennet, as demonstrated by milk clotting assays performed under various conditions of temperature, salt concentration, and pH[8-14].

Safety Concerns

FDA reviewed the safety of the enzyme preparation by assessing both

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the enzyme itself and the other components of the preparation. From the data discussed above, FDA concluded that the recombinant enzyme is virtually indistinguishable from the chymosin in rennet. Therefore, FDA concluded that the chymosin enzyme in the microbial preparation is as safe as that of the GRAS calf-rennet preparation.

The other components in the microbial preparation are the impurities derived from the production organism and the materials used in processing. Obviously, these impurities differ from those in a preparation derived from a calf stomach.

The determination that the manufacturing process does not introduce unsafe impurities into the final enzyme preparation was based primarily on the fact that chemicals used in the purification are approved food additives or GRAS substances. Additionally, the purification process destroys the production organism and removes most of the microbial material from the final product.

The isolation of chymosin from *E. coli* K-12, as described in published articles[8,9,13,14] and in the petition, takes advantage of the fact that the prochymosin proteins aggregate into insoluble inclusion bodies when overproduced in *E. coli*. After the cells are grown to the appropriate density in a fermentation vat, they are pelleted, washed, resuspended in a small volume, and lysed. The inclusion bodies are then collected and centrifuged at low speed, leaving in solution the bulk of the cellular material. After washing, the inclusion bodies are solubilized by denaturation in alkaline urea. The prochymosin is then renatured, further purified via anion exchange chromatography, and activated to chymosin via acid hydrolysis.

The production strain used by the petitioner carries an antibiotic resistance marker, the beta lactamase gene encoding ampicillin resistance. To ensure that the food-grade enzyme preparation would not contain functional copies of this gene, the petitioner added one further purification step to those described above—treating the purified inclusion bodies with acid prior to solubilizing them. This step is intended to destroy any unlysed cells pelleted with the inclusion bodies and to degrade any trapped DNA.

The resulting chymosin preparation is significantly purer than traditional rennet. According to data in the petition, some 60-80 percent of the total protein in the preparation is chymosin, as compared with only two percent in commercial rennet. Its use in food processing as a replacement for rennet would result in a very low daily intake, less than a half milligram per person, or 10 micrograms per kilogram body weight.

FDA based its conclusion that the production strain is safe for use in the production of a purified chymosin preparation primarily on published evidence demonstrating that it is nonpathogenic and nontoxigenic. Such evidence includes studies showing that *E. coli* K-12 does not colonize the gut of man or other animals even at high concentrations (10^9-10^{10} viable organisms per ingestion)[15-17], that the K-12 strain has been widely used as a laboratory organism for 30 years with no reported incidents of illness[15], that it does not produce toxins that cause illness upon ingestion[15,16], and that it is deficient in virtually all characteristics necessary for pathogenesis[15]. Additionally, nonpathogenic strains of *E. coli* are a part of the normal flora of the gastro-intestinal tract of man, where they are found at 10^6-10^8 organisms per gram of intestinal content[15].

Thus, even though it is not a common food-use organism and there have been no traditional feeding studies performed with it, FDA concluded that there is sufficient published information on *E. coli* K-12 to demonstrate that it is safe for producing chymosin.

As corroborative evidence of safety of its production strain, the petitioner submitted unpublished data from an *in vitro* test demonstrating that the strain produces no detectable shiga-like toxin, a potent enterotoxin produced by some pathogenic strains of *E. coli*. This was not surprising, since K-12 strains have not been found to contain the shiga-like toxin gene[18].

FDA ON PAPER

While the main article describes the details of FDA's approval of a specific food additive—chymosin—the information in this sidebar explains what FDA expects to see in a product application—and why.

Food Additives Amendment

In 1958, Congress passed the Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act. This amendment, Section 409 of the Act, mandates that food additives be approved by FDA before they may be used in food. Prior to this, FDA had the authority to remove adulterated substances from the food supply, but the burden was on the Agency to demonstrate that the substances were unfit or unsafe for consumption. With this amendment, Congress shifted the burden onto industry to demonstrate that a substance is safe before it may be marketed for use in food.

A food additive is defined, in part, as a substance whose intended use results directly or indirectly in its becoming a component of food or otherwise affecting the characteristics of food (21 USC 321[s]). However, substances that are generally recognized as safe (GRAS) are excluded from the definition. The general recognition of safety must be held by scientific experts, based either on publicly available scientific information or on evidence of common safe use in food prior to January 1, 1958.

A sponsor that wants to market a food additive must, in addition to ensuring that the product is of appropriate food grade, submit a petition to FDA containing information to establish that the substance is safe for its intended use and that it performs its intended function. FDA reviews the information and, if it finds the information adequate, publishes an authorizing regulation listing the substance and the use for which it is approved. Only then can the food additive be legally used in food.

GRAS food ingredients, not being considered food additives, are exempt from the premarket approval requirement. Indeed, many food ingredients that are accepted as GRAS because of their long and widespread use do not appear on any lists maintained by FDA.

However, it is prudent for sponsors to consult with FDA about the regulatory status of new products for which they believe adequate information exists to support a GRAS determination. This is also true for sponsors of traditional ingredients used in significantly new ways or manufactured by significantly new methods. The sponsor otherwise risks challenge from the Agency if it disagrees with his GRAS determination. Sponsors may submit, or may be requested to submit, a GRAS affirmation petition to enable FDA to fully evaluate the GRAS status of such a product.

GRAS affirmation petitions for new (post-1958) or newly made products must contain the same amount of scientific information as required for a food additive petition; additionally, to demonstrate the general recognition, they must document that the information critical to establishing the safety and function of the food ingredient is published. Thus, getting an ingredient affirmed as GRAS—that is, establishing that it is exempt from premarket approval—can be more arduous than obtaining that approval.

Presently, companies that have submitted petitions to FDA for substances produced using the newer methods of biotechnology have all requested that they be affirmed as GRAS. The products are enzymes derived from microorganisms into which DNA has been introduced with recombinant DNA techniques.

Safety Review of Biotech Products

Before approving the use of a food ingredient, whether produced by traditional or new techniques, FDA must find that a reasonable certainty exists that the product is safe at the levels it is expected to be consumed. When evaluating the safety of microbial enzyme preparations, for example, FDA evaluates the safety of both the enzyme and the impurities likely to be in the preparation. Because the impurities will derive from or be

To further substantiate the safety of the chymosin preparation, the petitioner submitted two unpublished short-term *in vivo* studies—a five-day feeding study in dogs and a one-month gavage study in rats. No adverse effects were observed in these studies at any dose tested. The petitioner also submitted data demonstrating that there was no detectable transformable DNA in the preparation, and no DNA fragments larger than 200 bases detectable by radiolabeled hybridization after gel electrophoresis. For comparison, the coding sequence of the antibiotic resistance gene carried by the production strain is 858 bases[19].

Additionally, the chemical and microbial purity of the enzyme preparation was in compliance with the requirements of the Food Chemicals Codex, 3rd Edition.

remnants of both the production organism and materials used in the manufacturing process, FDA examines the safety of the production organism, as well as the steps in its fermentation and in isolating, purifying, and stabilizing the final product.

If chemical, microbiological, or molecular biological information shows that the enzyme is the same as or substantially equivalent to an accepted food-use enzyme and that the impurities in the preparation raise no safety concerns then minimum toxicological testing may be necessary.

New substances, however, are likely to require more extensive toxicological testing, the nature of which would be based on considerations such as the identity of the product and the amount in the diet to which consumers would be exposed.

In assessing the safety of the production organism, FDA reviews a variety of factors to determine if the organism is well-characterized for its intended use and if it is pathogenic or toxigenic. For recombinant production organisms, this includes examining all steps in their construction to ensure that all vectors used are safe and that the inserted DNA does not encode unknown or potentially toxic proteins. The entire segment of cloned DNA, including sequences flanking the target gene, should be analyzed. If the donor organism produces toxins or other undesirable compounds, data should be provided demonstrating that DNA encoding these substances was not inadvertently cloned along with the target DNA.

Recombinant microorganisms frequently contain marker genes that encode resistance to therapeutically useful antibiotics. It is FDA's policy that enzyme preparations derived from antibiotic-resistant microorganisms should not contain levels of viable cells, transmissible vectors, or intact DNA that might serve as sources of antibiotic resistance for pathogens with which the preparations come into contact. Therefore, FDA reviews whether there is documentation that such transfer cannot happen at a biologically significant frequency.

Environmental Review

Under the National Environmental Policy Act (NEPA), FDA and other federal agencies must evaluate the potential environmental effects of their actions and include these evaluations in their decision-making. If the agency concludes that there will be significant effects, it must prepare a detailed environmental impact statement.

To fulfill its NEPA requirements, FDA evaluates whether the manufacture, including the use and disposal of all starting materials, and use of petitioned-for food ingredients could have a significant impact on the environment. For microbially-derived enzymes from recombinant strains, the focus of the environmental review centers around assessing the potential effects of the production organisms at the manufacturing site. The Agency examines many factors, such as whether the production organism has characteristics that would be expected to give it a competitive advantage in the environment; whether it is toxigenic or pathogenic or has other properties that would make it harmful to the environment; whether it contains mobile or easily mobilizeable plasmids or transposons that could transfer traits to other organisms; and the number of viable organisms likely to be incidentally released.

From the extent of its reviews so far, FDA has found that the organisms engineered to produce food-grade enzymes have low potential for causing significant environmental effects. The only characteristic that has raised questions is the presence of antibiotic resistance markers: will resistant production organisms incidentally released at the fermentation plant become established in the environment and become a reservoir for the spread of antibiotic resistance to human or domestic animal pathogens?

Petitioners have not relied on information aimed at directly answering whether the presence of particular antibiotic resistance markers would give host organisms a competitive advantage or would significantly contribute to the spread of antibiotic resistance in pathogens. Rather, the petitioners have focused on showing that the production strains have limited ability to survive and disperse in the environment; that the vectors present are nonconjugative and poorly mobilizeable; and, finally, that little or no release will occur because the production organisms are virtually all destroyed either during processing or prior to disposal from the fermentation plant.

Conclusions

After a comprehensive review of the information in the published literature, FDA concluded that the calf chymosin gene cloned into *E. coli* K-12 is expressed, that the gene product is chemically and biologically indistinguishable from calf chymosin, and that the impurities in the microbial chymosin preparation do not make the preparation unsafe. As for any food ingredient that is new or made by a new method, FDA reviewed the manufacturing method to determine product purity and identity.

From its review of this chymosin preparation and other new biotechnology-derived food ingredients, the Food and Drug Administraton is finding that the review of the safety, purity, and identity of these products is fundamentally no different from that of analogous products derived from unmodified or traditionally modified organisms. It is also finding that biotechnology provides powerful tools for resolving safety questions.

The author would like to thank Henry Miller, Buzz Hoffmann, Zofia Olempska-Beer, Eugene Coleman, Walter Hill, and especially James Maryanski for their comments and suggestions.

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G-2

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June 24, 2014

Via Electronic and U.S. Mail

Dr. Thomas Hendricks
Biologist
Ingredient Safety Team (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: AGRN 000-016 GRAS Notification for L-Methionine From a Modified *Escherichia coli* K-12

Dear Dr. Hendricks:

We are writing to inform you that Roquette Frères has transferred to Metabolic Explorer all rights, title, and interest in AGRN 000-016, the GRAS notification for L-methionine produced from a genetically modified *Escherichia coli* K-12 that was accepted by CVM for filing on January 3, 2014 and currently is under Agency review. As provided in the enclosed, there will be no change in the identity or manufacture of the product as described in AGRN 000-016 and MetEx reaffirms the claim of GRAS status for this product on the basis of the information and data provided in the Notification.

In light of this transfer, we hereby respectfully request that CVM replace Roquette Frères with "Metabolic Explorer" as the notifier of AGRN 000-016 and to list Metabolic Explorer as such in the Agency's Current Animal Food GRAS Notices Inventory. We also request that the Agency acknowledge in writing that Metabolic Explorer is now recognized by CVM as the notifier of this Notice.

For the Agency's records, we provide below the details for the contact at Metabolic Explorer:

Mr. Antoine Darbois
Secrétaire Général
Biopole Clermont Limagne
63 360 Saint-Beauzire
France
Telephone: +33 (0)4 73 33 43 00
E-mail: adarbois@metabolic-explorer.com

Please note that Metabolic Explorer has retained Keller and Heckman LLP to act on its behalf in matters relating to AGRN 000-016, so please continue to contact me if you should have any questions or concerns as the Agency completes its review of the notice.

We trust that this letter and its enclosure form a sufficient basis for the Agency to proceed with this request. Should you need additional information, or have any questions, please do not hesitate to contact us, preferably by electronic mail or telephone so that we can respond as quickly as possible.

Sincerely yours,

Mel Drozen

Melvin S. Drozen

Enclosure

cc: Mr. Geoffrey Wong (via email only)

Dr. Thomas Hendricks
Biologist
Ingredient Safety Team (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855
USA

Saint-Beauzire, on June 24th, 2014

RE : AGRN 000-016 GRAS Notification for L-Methionine From a Modified Escherichia coli K-12
Our ref. : AD/NF 2014-062401

Dear Dr. Hendricks,

The purpose of this letter is to inform you that Roquette Frères has transferred to Metabolic Explorer all rights, title, and interest in AGRN 000-016, the GRAS notification for L-methionine produced from a genetically modified *Escherichia coli* K-12, which currently is under Agency review.

We affirm that all information and data provided in AGRN 000-016 is complete and accurate, and that there will be no change to the identity of the notified substance or its method of manufacture, including the described genetic modifications to the *Escherichia coli* K-12. Consequently, we reaffirm the claim of GRAS status included in AGRN 000-016:

> Metabolic Explorer has determined that the use of L-methionine 85%, produced by a genetically modified Escherichia coli K-12, for use as a nutrient in animal feed is Generally Recognized as Safe based on scientific procedures, and is thus exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 *et seq.*).

AD

We trust that this letter forms a sufficient basis for the Agency to transfer the rights to AGRN 000-016 to Metabolic Explorer. Should you need additional information, or have any questions, please do not hesitate to contact Mel Drozen of Keller and Heckman LLP on our behalf.

Cordially yours,

Antoine Darbois
Secrétaire Général
Metabolic Explorer

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T-3

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October 8, 2014

Via Electronic Mail and Federal Express

CONTAINS CONFIDENTIAL BUSINESS INFORMATION

Dr. Thomas Hendricks
Biologist
Ingredient Safety Team (HFV-224)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: Supplemental Information on AGRN 000-016 GRAS Notification for L-Methionine From a Modified *Escherichia coli* K-12

Dear Dr. Hendricks:

The purpose of this letter is to respond, on behalf of our client, Metabolic Explorer (MetEx), to the U.S. Food and Drug Administration Center for Veterinary Medicine's (CVM) requests for information and clarification relating to AGRN 000-016, as communicated during our teleconference on September 2, 2014. This letter and its enclosures provide a full response to each of the requests. Please note that this letter and the attachments contain confidential information that, in accordance with the Agency's public information regulations, should not be released to third parties.

Before turning to our responses, we wanted to let you know that MetEx intends to submit

(b) (4)

[redacted]

(b) (4)

We turn now to our responses to CVM's questions. For ease of reference, we repeat below the requests discussed during our September 2, 2014 call, followed by our responses.

1) CVM requested data to demonstrate that the isomer of L-methionine 85% has the L- configuration, not the D- configuration, as L-methionine is more bioavailable. Specifically, CVM requested that the notifier provide at least two chromatograms of the L-methionine 85% product along with at least one chromatogram of an L-methionine standard for comparison. CVM indicated that NMR or mass spectrometry analyses would be suitable, provided the L-isomer can be distinguished from the D- isomer.

To demonstrate that L-methionine 85% is comprised of the L-isomer of methionine, in Figure 1, below, we provide two HPLC chromatograms of the L-methionine 85% (labeled as L-Methionine Batch003), along with chromatograms of L-methionine and D-methionine standards. Figure 2 presents an overlay of all four runs presented in Figure 1 to visually demonstrate that the peaks associated with the L-methionine 85% match the L-methionine standard peak, not the D-methionine peak.

The enantiomeric purity of L-methionine 85% was analyzed by HPLC using a

(b) (4)

Figure 1: Individual chromatograms of two L-methionine 85% runs (red with retention time of 42.279 min and purple with the retention time of 42.271 min), L-methionine standard (blue with the retention time of 42.339 min), and D-methionine (brown with the retention time of

53.365 min).

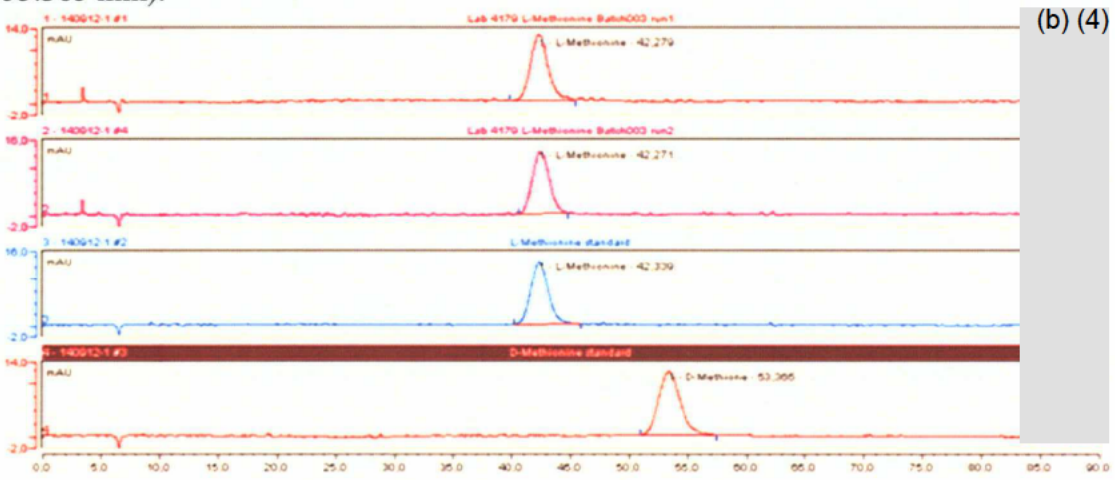
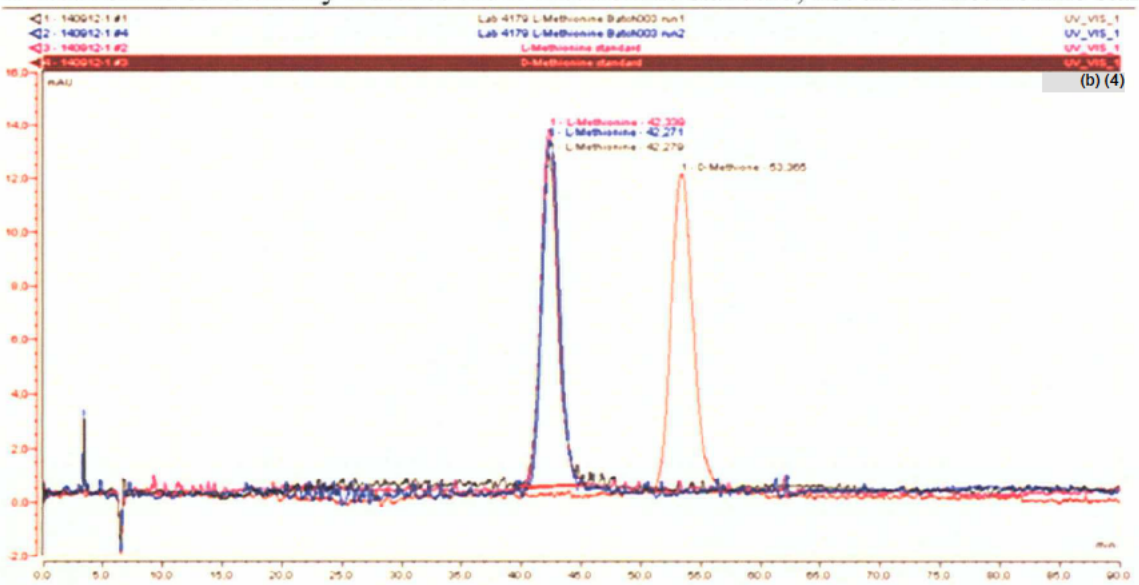


Figure 2: Chromatograms from Figure 1 overlapped for comparison to demonstrate that L-methionine 85% clearly matches the L-methionine standard, not the D-methionine standard.



2) CVM requested information describing why the (b) (4)

substances used in the growth medium are appropriate for animal feed uses.

As described in the GRAS Notification, there are (b) (4) different formulations for the growth media used during the preculture and fermentation processes that produce the L-

methionine 85%, (b) (4). Of the components of the growth media identified in the Notification, CVM has requested information supporting a conclusion that the (b) (4) are safe for use in animal feed applications.

We first note that it is highly unlikely that any of these components would be present in the finished L-methionine due to the separation and purification processes that are used to extract the L-methionine from the *E. coli* K-12 and produce the finished product. These substances are components of the growth medium that the *E. coli* uses to produce L-methionine. Other than the (b) (4) all of these components are nutrients for the *E. coli* K-12, providing sources of (b) (4). As such, they will be consumed and metabolized by the *E. coli* during the fermentation process. The (b) (4) is used as (b) (4) Thus, they would not be incorporated into the L-methionine. Once the fermentation process is complete, the *E.coli* K-12 cells containing the L-methionine are (b) (4). These steps are fully described in the Notification and demonstrate the slim possibility that any of these components, present in the growth medium as (b) (4), would be present in the finished L-methionine product.

We also note that as part of the Notice, a complete mass-balance analysis of the finished L-methionine product was provided. This analysis included (b) (4). From this analysis, we can conclude that (b) (4) are present in the L-methionine 85% at low levels that present no safety concern, as described in the Notice.

(b) (4)

(b) (4)

For these reasons, we can conclude that the growth medium components identified by FDA either will not be present in the finished L-methionine 85% product or are present at levels that present no realistic risk of any safety concern, and thus the growth medium and (b) (4) are safe for their intended use as described above and in the Notification.

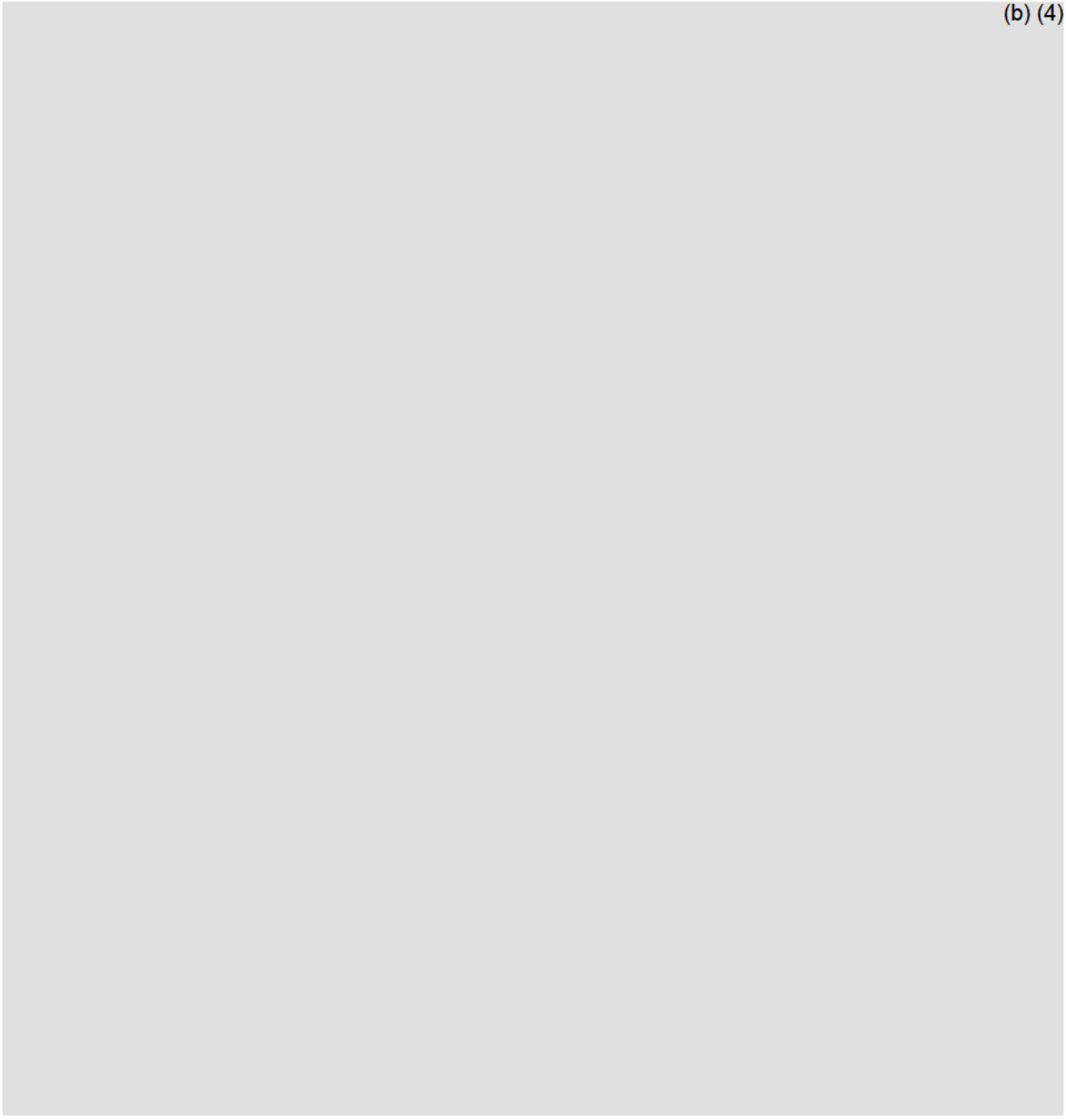
3) CVM requested additional information regarding the promoter used in

(b) (4)

.

(b) (4)

(b) (4)



5) CVM requested information regarding the commercial container intended to package the L-methionine 85%.

(b) (4) and (b) (4) are currently the expected materials to be used to sell L-methionine 85%. The packaging material used will depend on (b) (4).

6) CVM asked whether there is an intended expiration date for the L-methionine 85%. CVM noted that in Attachment 7 of the notice, the stability studies concluded that 24 months of storage at 25°C under 60% relative humidity (RH) and 18 months at 40°C under 75% relative humidity showed no significant decrease in the rate of L-methionine. CVM asked whether the conditions and times described in the stability studies dictate the intended expiration dates.

(b) (4)

* * *

We trust that the information provided above and in the attachments are sufficient to address CVM's questions. Of course, should you have any additional questions, please do not hesitate to contact us, preferably by e-mail or telephone, so that we may respond as quickly as possible. Thank you for your assistance in this matter.

Sincerely yours,

Melvin S. Drozen

Enclosures

cc: Mr. Geoffrey Wong (via email only)

T-5

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November 20, 2014

Via Electronic Mail

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CONTAINS CONFIDENTIAL BUSINESS INFORMATION

Re: Second Supplemental Information on AGRN 000-016 GRAS Notification for L-Methionine From a Modified *Escherichia coli* K-12

Dear Dr. Hendricks:

The purpose of this letter is to respond, on behalf of our client, Metabolic Explorer (MetEx), to the U.S. Food and Drug Administration Center for Veterinary Medicine's (CVM) requests for information and clarification relating to AGRN 000-016, as communicated in your email to us on November 4, 2014. This letter provides a full response to CVM's request to compare the open reading frame sequences to annotated protein sequences found in publically available repositories. Please note that this letter contains confidential information that, in accordance with the Agency's public information regulations, should not be released to third parties.

For ease of reference, we repeat the email correspondence sent on November 4, 2014, followed by our response.

We are contacting you in regards to AGRN 000-016 (GRAS notification submitted by Metabolic Explorer for L-methionine 85%). There appears to be a misunderstanding with respect to the type of information that we were requesting from the ORF analysis. An open reading frame analysis is used to determine whether insertion of a nucleotide sequence into the host's genome could result in the production of proteins that may raise safety concerns. The information provided in your submission indicates that there are (b) (4)

In the past researchers and companies have addressed this by comparing the

annotated protein sequences against protein sequences that comprises GenBank or Uniprot repositories. Please summarize the results of this type of analysis for the (b) (4) .

In Attachment 1 to this letter, we provide our analysis comparing the open reading frame protein sequences (originally provided to CVM as Attachment 5 to our letter of October 8, 2014) to those found at GenBank (http://www.ncbi.nlm.nih.gov/genbank). As you know, open reading frame (ORF) analysis searches and identifies stretches of DNA between a start codon (typically ATG, and in a few cases GTG, TTG, or CTG) and a stop codon (TAA, TGA, or TAG). An ORF analysis does not indicate whether these stretches of DNA will be expressed into proteins, potentially resulting in the generation of false positives.

As *E. coli* K-12 is a model laboratory microorganism whose genome has been sequenced and analysed since 1997,[1] many, although not all, of the expressed protein sequences have been identified and described in publically available protein databases, especially in comparison to other microorganisms. (b) (4) .

(b) (4)

The analyses from the MvirDB toxin databases demonstrate that there are were no significant matches to any known toxins considering the following:

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1 Blattner, et al. "The complete genome sequence of Escherichia coli K-12" *Science,* 277: 5331 (1997), 1453-1462.

2 Zhou, C. E., et al. "MvirDB—a microbial database of protein toxins, virulence factors and antibiotic resistance genes for bio-defence applications." *Nucleic Acids Research,* 35: 1 (2007), D391-D394, available at http://nar.oxfordjournals.org/content/35/suppl_1/D391.full.pdf+html.

1) The majority of the results showed a high "E-value," which indicates that the hits are not statistically significant and instead are highly likely to be attributed to random chance;

2) The length of amino acids (match length) homologous to toxin sequences were very short; and

3) Of those short matching amino acid sequences, there was a low percentage of identities within the match length, often less than 50%.

Given that the matches were short, with a low percentage identities, and with a high E-value, it is reasonable to conclude that none of the ORF sequences were a significant match to any known toxin, and thus present no health or safety concern to animal or human.

We trust that we have fully responded to your November 5 request. Please let us know if you have any further questions.

Sincerely yours,

Mel Drozen

T-6

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January 15, 2015

Via Electronic Mail and FedEx

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Supervisory Interdisciplinary Scientist
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Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: Supplemental Information on AGRN 000-016 GRAS Notification for L-Methionine From a Modified *Escherichia coli* K-12

Dear Mr. Wong:

This letter responds, on behalf of our client, Metabolic Explorer (MetEx) to your request for information relating to AGRN 000-016, as communicated during our teleconference on January 12, 2015.

First, in response to your request regarding the regulatory status of the components used in the manufacture of the L-methionine 85%, we can confirm that all of the substances, including (b) (4), used to manufacture L-methionine 85% intended for addition to animal feed are safe and suitable for their intended use because they are in compliance with a regulation of the Food and Drug Administration, a listing in the Official Publication of the American Association of Feed Control Officials, or have been determined by Metabolic Explorer to be generally recognized as safe (GRAS) for the intended use.

In response to your second request, we believe it is reasonable to conclude that when L-methionine 85% is mixed with the animal feed and, potentially, pelletized, that it will mix in a homogenous manner. We first note that there is no L-methionine product currently commercially available in either the United States or European Union. Therefore, it is not possible to directly compare the physical or chemical characteristics of L-methionine 85% to a "standard" L-methionine product. DL-methionine is commercially available in the U.S., and although MetEx has not directly compared the physical characteristics of DL-methionine products currently on the market to L-methionine 85%, there is no reason to believe that the two

substances, which both would be used in powdered form, would behave differently when mixed into animal feed and pelletized.

In support of this conclusion, we look to efficacy testing that was conducted on the L-methionine 85% as compared to commercially available DL-methionine, when both products were mixed into animal feed, pelletized, and fed to weaner pigs. In that testing, L-methionine 85% and DL-methionine were mixed into the feed at varying dose levels and the supplemented feed was pelletized. Feed samples were chemically analyzed to determine their composition, including amino acid content and, specifically, free methionine. At the different dose levels, the levels of free methionine in both the DL-methionine and L-methionine 85% test diets were very similar, supporting the conclusion that L-methionine 85% mixes into animal feed and is pelletized adequately and in the same manner as DL-methionine. On that basis, we further conclude that L-methionine 85% will demonstrate acceptable homogeneity in mixing studies and acceptable pelleting stability.

We trust that the information provided above will be sufficient to permit FDA to proceed quickly to providing the no questions letter. Please let us know as soon as possible if anything further is needed to close out the Agency's review. Thank you for your assistance in this matter.

Sincerely yours,

Mel Drozen

Melvin S. Drozen

cc: Dr. Thomas Hendricks (via email only)
Ms. Chelsea Trull (via email only)