

GRAS Notice (GRN) No. 523

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

**ORIGINAL SUBMISSION**

NutraSource, Inc.  
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May 20, 2014

GRN 000523

Dr. Antonia Mattia  
Office of Food Additive Safety (HFS-255)  
Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5 100 Paint Branch Parkway  
College Park, MD 20740

Subject: GRAS notice for L-Leucine

Dear Dr. Antonia Mattia:

On behalf of InnoBio<sup>®</sup> Limited (InnoBio<sup>®</sup>), we are submitting a GRAS notification for L-Leucine for FDA review. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance. We believe that this determination and notification are in compliance with proposed Sec. 170.36 of Part 21 of the Code of Federal Regulations (21 CFR section 170.36) as published in the Federal Register, Vol. 62, No. 74, FR 18937, April 17, 1997.

We enclose an original and two copies of this notification for your review. Please feel free to contact me if additional information or clarification is needed as you proceed with the review. We would appreciate your kind attention to this matter.

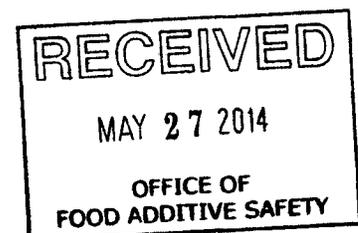
Sincerely,

(b) (6)

Susan Cho, Ph.D.  
Susanscho1@yahoo.com  
Agent for InnoBio<sup>®</sup>

5/20/2014

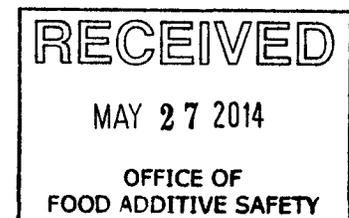
enclosure



Leucine GRAS

GRAS Notification for L-LEUCINE  
produced by INNOBIO<sup>®</sup> Limited (INNOBIO<sup>®</sup>)  
GRN 000523

Prepared by: NutraSource, Inc.  
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## Abbreviations

21 CFR = Title 21 Code of Federal Regulations  
ACE-I =angiotensin-converting enzyme inhibitor  
ASN = American Society for Nutrition  
AUC = area under the curve  
BCAT = branched-chain aminotransferase  
BCAA = branched amino acids  
BCKDH = branched-chain alpha-ketoacid dehydrogenase  
BHBN = N-butyl-N (4-hydroxybutyl) nitrosamine  
cGMP = current Good Manufacturing Practice  
CRP = C reactive protein  
*E. coli* = *Escherichia coli*  
EAR = estimated average requirements  
EDI = estimated daily intakes  
EDL = extensor digitorum longus  
EU = European Union  
FCC=Food Chemicals Codex  
FDA = Food and Drug Administration  
FEMA = Flavor and Extract Manufacturers' Association  
GRAS = generally recognized as safe  
HACCP = hazard analysis and critical control point  
HCC=hepatocellular carcinoma  
HCV=hepatitis C virus  
HE=hepatic encephalopathy;  
HFD = high fat diet  
HOMA-IR = homeostatic insulin resistance  
ICAAS = International Council for Amino Acid Sciences  
IL = interleukin  
IOM = Institute of Medicine  
IPGTT = intraperitoneal glucose tolerance test  
KIC = alpha-ketoisocaproate  
LNAAs = large neutral amino acids  
LDL-C = low density lipoprotein cholesterol  
LOAEL = lowest observed-adverse-effect level  
M=males  
MDA = malondialdehyde  
MeHis = 3-methylhistidine  
MI = myocardial infarction  
mTOR = mammalian target of rapamycin  
NAS = National Academy of Sciences  
NHANES = National Health and Nutrition Examination Survey  
NEFA = non-esterified fatty acid  
NOAEL = no observed-adverse-effect level  
NRC = National Research Council  
P = parallel

## Leucine GRAS

PDRNS = Physicians' Desk Reference for Nutritional Supplements

RACC = reference amounts customarily consumed

RFA=radiofrequency ablation

RNA = ribonucleic acid

SCEs = sister chromatid exchanges

T2DM=Type 2 Diabetes Mellitus

TC = total cholesterol

TG = triglycerides

TGF- $\beta$ = transforming growth factor- $\beta$

TNF $\alpha$  = Tumor necrosis factor alpha

UL= tolerable upper intake levels

USDA= United States Department of Agriculture

X=crossover



## I. GRAS EXEMPTION CLAIM

### I.A. L-Leucine of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 5 170.36(1)

INNOBIO® Limited (INNOBIO®; the notifier) has determined that its Vegan Instantized L-Leucine Powder is Generally Recognized As Safe (GRAS). Consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*, this determination is based on scientific procedures described in the following sections. Since these procedures specify the conditions of its intended use in food, the use of L-leucine is exempt from the requirement of premarket approval.

Signed

(b) (6)

5/20/2014

Susan Cho  
Agent for  
INNOBIO® Limited (INNOBIO®)

Date:

### I.B. Notifier's Name and Address

Notifier's name: INNOBIO® Limited (INNOBIO®)

Phone number: 86-411-87406671

Fax number: 86-411-87406801

Physical address: No.49, DDA, Dalian, Liaoning, 116600, China

E mail address:sunx@INNOBIO.cn

### I.C. Name of GRAS Substance

The common name of the notified substance is L-leucine: INNOBIO® Vegan Instantized L-Leucine Powder.

### I.D. Product Description

L-leucine is an indispensable amino acid or essential amino acid, whose skeleton cannot be synthesized from simpler molecules in humans, and therefore must be ingested. L-leucine, along with L-isoleucine and L-valine, are classified as branched chain amino acids (BCAA) that differ from most other indispensable amino acids in that enzymes initially responsible for their catabolism are found primarily in extrahepatic tissues. L-leucine is also classified as a hydrophobic amino acid due to its aliphatic isobutyl side chain and is a major component of the subunits in ferritin, astacin and other 'buffer' proteins.

Supplements of L-leucine and the BCAA are advocated and widely used in relation to performance in various clinical situations to maintain muscle mass during weight loss and in the elderly (Millward, 2012). Muscle mass is regulated by the synthesis and degradation of muscle protein, which in turn is affected by aging, several catabolic diseases, and malnutrition. Amino acids, particularly leucine, are known to stimulate muscle protein synthesis and suppress muscle protein degradation (Sugawara et al., 2007). Thus, L-leucine has earned more attention on its own as a catalyst for muscle growth and muscular insurance.

The estimated average requirement (EAR) of L-leucine is 34 mg/kg body weight/day which may correspond to 2.38 g/day for a 70 kg adult (Institute of Medicine [IOM], 2005). The IOM has not established Tolerable Upper Intake Levels (UL) for L-leucine and other indispensable amino acids. The UL is defined as the highest average daily intake of a nutrient that is likely to pose no risk of adverse health effects for nearly all persons in the general population. Recently, the International Council for Amino Acid Sciences (ICAAS) has proposed an upper limit of safe intake for L-leucine for healthy adults as 0.53 g/kg/day, which corresponds to 37.1 g/day in a 70 kg adult.

## **I.E. Applicable Conditions for Use of the Notified Substance**

### **I.E.1. Current Regulatory Status and Background Dietary Intake of L-Leucine**

L-Leucine, free, hydrated, or anhydrous or as the hydrochloride, sodium or potassium salts, is permitted for use as a special dietary and nutritional additive to significantly improve the biological quality of the total protein in a food containing naturally occurring primarily-intact protein that is considered a significant dietary protein source (Title 21 Code of Federal Regulations [21 CFR] 172.320) (U.S. Food and Drug Administration [FDA], 2009). As a food additive, L-leucine should meet the specifications established by the Food Chemicals Codex (FCC), National Academy of Sciences/National Research Council (NAS/NRC), and the amount of L-leucine added for nutritive purposes plus the amount naturally present in free and combined (as protein) form should not exceed 8.8% by weight of the total protein of the finished food. L-Leucine also may be used as a lubricant in the manufacture of aspartame or neotame tablets for sweetening hot beverages at levels not to exceed 3.5% of the tablet weight (21 CFR 172.804; 21 CFR 172.829; U.S. FDA, 2009). Additionally, L-leucine is deemed to be GRAS by the Flavor and Extract Manufacturers' Association (FEMA) under specific conditions of use.

### **I.E.2. Intended Use of L-Leucine and Levels of Use in Foods**

INNOBIO<sup>®</sup> intends to market L-leucine as a food ingredient in selected food categories, including milk and non-milk based meal replacements, sports and isotonic beverages, vitamin enhanced waters, and meal replacement bars at levels that provide 0.5 to 3 g L-leucine/serving.

### **I.E.3. Estimated Dietary Intakes (EDIs) of L-Leucine Based upon Intended Food Uses**

L-Leucine occurs in the diet as a component of protein. In particular, dairy products are a rich source of L-leucine (United States Department of Agriculture [USDA], 2006). The Americans' mean and 90th percentile intakes of L-leucine from diets, without considering the intended use, were estimated to be 6.08 and 8.90 g/person/day, respectively (IOM, 2005). The highest 90th percentile intake of L-leucine was reported in men ages 19 to 30 years at 11.1 g/person/day (IOM, 2005).

The intended uses of L-leucine are the same as those described in GRN 308 and will substitute for currently marketed L-leucine products. L-Leucine is proposed for use as an ingredient in milk and non-milk based meal replacements, sports and isotonic beverages, vitamin enhanced waters, and meal replacement bars, at levels of 0.5-3.0 g/serving. Consequently, INNOBIO<sup>®</sup> notes that its uses will not result in any exposure beyond what was previously estimated. Based on previous analysis of National Health and Nutrition Examination Survey (NHANES) 2005-2006 (CDC, 2006), the proposed food uses would result in an estimated daily intake (EDI) for users

## Leucine GRAS

(mean of 1.9 g/person/day or 28 mg/kg body weight/day and 90th percentile intake of 4.1 g/person/day or 64 mg/kg body weight/day) at levels significantly below those associated with any potential side effects (GRN 308; U.S. FDA, 2010) In addition, these estimates are highly optimistic since it is not likely that L-leucine will be used at maximum ratios for all food categories under the intended uses. Also, food wastes should be considered. Overall, intended use will result in EDIs at levels significantly below those associated with any potential side effects.

### **I.E.4. Basis for the GRAS Determination**

Pursuant to 21 CFR 9170.35, L-leucine has been determined by INNOBIO® to be GRAS on the basis of scientific procedures (U.S. FDA, 1997, 2009). This determination is based on the views of experts who are qualified by scientific training and experience to evaluate the safety of L-leucine as a component of food. Expert panel members, Susan Cho, Ph.D., Paul Pencharz, M.D., Ph.D., and, Steven Heymsfield, M.D., have critically reviewed and evaluated the publicly available information summarized in this document and have individually and collectively concluded that L-leucine, produced consistent with current Good Manufacturing Practice (cGMP) and meeting the specifications described herein, is safe under its intended conditions of use. It is also INNOBIO's opinion that the proposed use of L-leucine is not only safe within the terms of the Federal Food, Drug, and Cosmetic Act (meeting the standard of reasonable certainty of no harm), but it is also GRAS according to 21 CFR according to consensus among experts. The Panel further unanimously concludes that these uses of L-leucine are GRAS based on scientific procedures, and that other experts qualified to assess the safety of food and food ingredients would concur with these conclusions.

### **1.F. Availability of Information**

The detailed data and information that serve as a basis for this GRAS determination will be provided to the U. S. FDA upon request, or are available for the FDA's review and copying during reasonable business hours at the offices of NutraSource:  
6309 Morning Dew Ct., Clarksville, MD 21029, USA.

## II. INFORMATION ABOUT THE IDENTITY OF THE NOTIFIED SUBSTANCE

### II.A. Common or Trade Name

The common name of the notified substance is L-leucine: INNOBIO<sup>®</sup> Vegan Instantized L-leucine Powder.

### II.B. Chemical Description

INNOBIO's L-leucine product comprises not less than 98.0% L-leucine. L-Leucine occurs as white powder with a slightly bitter taste. L-Leucine is freely soluble in formic acid, sparingly soluble in water, and relatively insoluble in ethanol.

### II.C. Standards of Identity

INNOBIO<sup>®</sup> intends to use L-leucine as an ingredient in several food categories, including those that have standards of identity located in Title 21 of the Code of Federal Regulations. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if permitted by the applicable standard of identity in Title 21 of the Code of Federal Regulations.

### II.D. Background

L-leucine is an indispensable amino acid or essential amino acid, whose skeleton can not be synthesized from simpler molecules in humans, and it therefore must be ingested. L-leucine, along with L-isoleucine and L-valine, are classified as BCAA that differ from most other indispensable amino acids in that enzymes initially responsible for their catabolism are found primarily in extrahepatic tissues. L-leucine is also classified as a hydrophobic amino acid due to its aliphatic isobutyl side chain and is a major component of the subunits in ferritin, astacin and other 'buffer' proteins.

Leucine is utilized in the liver, adipose tissue, and muscle tissue. In adipose and muscle tissue, L-leucine is used in the formation of sterols. The combined usage of L-leucine in these two tissues is seven times greater than its use in the liver (Rosenthal et al., 1974).

Table 1 presents Estimated Average Requirements (EAR) of L-leucine established by the Institute of Medicine (IOM; 2005). The EAR of L-leucine is 34 mg/kg body weight/day which corresponds to 2.38 g/day for a 70 kg adult. However, a recent research indicates that an appropriate EAR for adults is 55 mg/kg body weight/day, which corresponds to 3.85 g/day for a 70 kg adult (Elango et al., 2012a).

Table 1. EAR for L-Leucine, mg/kg body weight/day

	EAR <sup>1</sup>	EAR <sup>2</sup>
Adults	34	55
Young children, 1-3	48	--
Young children 4-6 y	40	--
Boys, 9-13y	40	--
Girls, 9-13 y	38	--
14-18 y, boys	38	--
14-18 y, girls	35	--

Source: <sup>1</sup>IOM, 2005; <sup>2</sup>Elango et al., 2012a.

L-leucine has the capacity to stimulate muscle protein synthesis (Etzel, 2004). Supplements of L-leucine and the BCAA are advocated and widely used in relation to performance and in various clinical situations to maintain muscle mass during weight loss and in the elderly (Millward, 2012). Muscle mass is regulated by the synthesis and degradation of muscle protein, which in turn is affected by aging, several catabolic diseases, and malnutrition. Amino acids, particularly leucine, are known to stimulate muscle protein synthesis and suppress muscle protein degradation (Sugawara et al., 2007).

The IOM has not established UL for L-leucine and other indispensable amino acids. The UL is defined as the highest average daily intake of a nutrient that is likely to pose no risk of adverse health effects for nearly all persons in the general population. It is intended to specify the level above which the risk for harm begins to increase. As intake increases above the UL, the potential risk for adverse effects increases. In short, the UL is a reference value intended to guide policy-makers and scientists charged with ensuring a safe food supply and protecting the health of Americans. In stating the reasons why the ULs have not been established for individual amino acids, the IOM states the following:

- "There is no evidence that amino acids derived from usual or even high intakes of protein from food stuffs present any risk."
- "More emphasis was placed on observations of adverse effects in humans than on effects observed in animals, and pharmacokinetic studies were sought to bridge potential differences between animals and humans."
- "Many animal studies of amino acid toxicity were conducted with diets deficient in protein. Less emphasis was placed on these studies than those with adequate protein diets because of creation of amino acid imbalances."
- "For some well studied amino acids, there were no adverse effects reported at the highest dose tested in long term studies. In such cases it was not possible to establish a Lowest Observed Adverse Effect Level (LOAEL) or a No Observed Adverse Effect Level (NOAEL) that was supported by toxicity data. Under these circumstances it was not possible to establish a UL in keeping the criteria and procedures required by the UL model."

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Recently, the International Council for Amino Acid Sciences (ICAAS) has proposed an upper limit of safe intake for L-leucine for healthy adults as 0.53 g/kg body weight/day, which corresponds to 37.1 g/day in a 70 kg adult (Cynober et al., 2012; Elango et al., 2012b; Pencharz et al., 2012; Pencharz and Russell, 2012).

### II.E. Chemistry, Physico-Chemical Properties, and Structure

Chemical Name: (2S)-2-amino-4-methylpentanoic acid; L-2-amino-4-methylvaleric

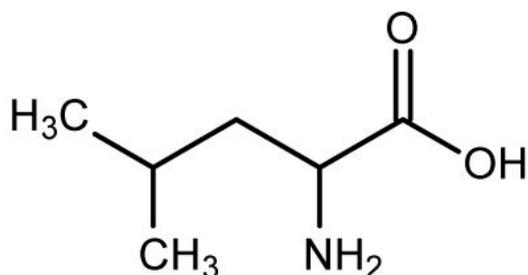
Chemical Abstracts Service Number: 61 -90-5

Empirical Formula and Formula Weight: C<sub>6</sub>H<sub>13</sub>NO<sub>2</sub>

Molecular Weight: 131.17 g/mol

Figure 1 shows chemical structure of L-leucine.

Figure 1. Structure of L-Leucine



### II.F. Manufacturing Process

INNOBIO's Vegan Instantized L-leucine is manufactured under current Good Manufacturing Practice (cGMP) production and purification.

L-Leucine is obtained via fermentation of glucose and corn steep liquor using *Corynebacterium glutamicum* which has been safely used in industrial production of amino acids for a long time. For L-leucine production, sterilized glucose and corn steep liquor are fermented with a non-pathogenic, non-toxic species and strain of *Corynebacterium glutamicum* (MD0032) at 28-30°C for 2-3 days in a fermentation tank. After fermentation, the broth is passed through an ion exchange resin. The filtrate is concentrated and decolorized. Food grade lecithin and water are added to decolorized L-leucine concentrate. This mixture is emulsified in a high-speed shear mixer and is dried at 80~120°C to obtain a dried powder. The dried powder is passed through a sieve (40 mesh) to obtain Vegan Instantized L-leucine powder. All equipment and materials used in the production process have a safe history of use in food processing.

Process tanks and lines are cleaned with sodium hydroxide and hydrogen peroxide following standard procedures common to the dairy industry. All ion exchange resins used for chromatographic purification comply with 21 C.F.R. § 173.25. Celite is cleared under 27 CFR § 24.243 (Filtering aids). Similar uses of activated carbon are considered GRAS for purification

## Leucine GRAS

and clarification of wine as per 27 CFR §24.246. All processing aids used in the manufacturing process are considered safe and suitable.

### II. G. Specifications of INNOBIO<sup>®</sup>'s L-Leucine

The product specifications for INNOBIO<sup>®</sup> Vegan Instantized L-Leucine Powder are presented in Table 2 and include physical and chemical specifications. Certificates of analysis for 3 non-consecutive lots of L-leucine are presented in Appendix C.

Table 2. Specifications of INNOBIO<sup>®</sup> Vegan Instantized L-Leucine Powder

Items	Specifications	Methods
Appearance	White to off-white fine powder	Visual
L-Leucine Content (%)	98.0 Min.	HPLC
Bulk Density (g/ml)	0.30-0.60	USP 616
Particle Size	95% through 40 mesh	USP 786
Loss on Drying (%)	1.0 Max.	USP 731
Residue on Ignition (%)	0.40 Max.	USP 281
Lead (ppm)	1.0 Max.	EN ISO 17294
Cadmium (ppm)	1.0 Max.	EN ISO 17294
Hg (ppm)	1.0 Max.	EN ISO 17294
Arsenic (ppm)	1.0 Max.	EN ISO 17294
Total Plate Count (CFU/g)	3,000 Max.	ISO 4833
Yeasts and Molds (CFU/g)	100 Max.	ISO 7954
<i>Coliform</i> (CFU/g)	10 Max.	ISO 4832
<i>Escherichia coli</i>	Negative	ISO 16649
<i>Salmonella</i>	Negative/25g	ISO 6579
<i>Staphylococcus aureus</i>	Negative/g	ISO 6888

#### II.G.1. Stability of L-Leucine Preparation

INNOBIO<sup>®</sup> Vegan Instantized L-Leucine Powder is stable for up to 2 years at ambient temperature.

### III. INTENDED USES AND EXPOSURE ESTIMATES

#### III.A. Food Sources of L-Leucine

Table 3 presents L-leucine content in foods (USDA, 2006).

Table 3. L-Leucine Content in Foods

Leucine food sources	Leucine content, %
Soybeans, mature seeds, raw	2.97
Lentils, raw	2.03
Cowpea, catjang, mature seeds, raw	1.83
Beef, round, top round, separable lean and fat, trimmed to 1/8" fat, select, raw	1.76
Beef, top sirloin, separable lean only, trimmed to 1/8" fat, choice, raw	1.74
Peanuts, all types, raw	1.67
Salami, Italian, pork	1.63
Fish, salmon, pink, raw	1.62
Chicken, broilers or fryers, thigh, meat only, raw	1.48
Nuts, almonds	1.47
Egg, yolk, raw, fresh	1.40
Chickpeas (garbanzo beans, bengal gram), mature seeds, raw	1.37
Seeds, sesame butter, tahini, from raw and stone ground kernels	1.36
Chicken, broilers or fryers, wing, meat and skin, raw	1.29
Milk, sheep, fluid	0.59
Milk, whole, 3.25% milkfat	0.27

Source: USDA National Nutrient Database for Standard Reference, Release 19, 2006.

#### III.B. Intended Use

Table 4 shows the maximum use levels for L-leucine under the intended use.

Table 4. Summary of Maximum Use Levels for L-Leucine Under the Intended Use

Food category	Proposed food uses	Use level, g of added L-leucine/RACC	RACC, mL or g	Use level, % of added L-leucine
Beverages and beverage bases	Non-milk based meal replacements	1.5-3.0	240	0.625 to 1.25
	Sports and isotonic beverages	0.5	240	0.208
	Vitamin enhanced waters	1.0	240	0.416
Grain based products	Meal replacement bars	3.0	40	7.5
Milk products	Milk-based meal replacements	1.5-3.0	240	0.625 to 1.25

RACC = Reference Amounts Customarily Consumed per eating occasion (refers to the amount of the food or beverage product consumed).

### **III.C. Self Limiting Levels of Use**

The use of L-leucine is limited by its slightly bitter taste. Thus, the use of L-leucine in foods at upper use levels is largely self-limiting based on its organoleptic properties.

### **III.D. Exposure Estimates**

#### **III.D.1. EDIs Before the Intended Use**

Dairy products are a rich source of L-leucine which occurs in the diet as a component of protein (USDA, 2006). The IOM (2005) estimated that the mean and 90th percentile total population background dietary intakes of L-leucine in Americans are 6.08 and 8.90 g/person/day, respectively and that the highest 90<sup>th</sup> percentile intakes of L-leucine was in men ages 19 to 30 years at 11.1 g/person/day. British adults had similar intake patterns. In British adults, median and 90th percentile intake values for L-leucine were 6.5 and 8.3 g/d for 60 kg body weight (108 and 138 mg/kg body weight/day) or 8.3% of the protein intake (Millward et al., 2012).

#### **III.D.2. EDIs Under the Intended Use**

The intended uses of L-leucine are the same as those described in GRN 308 (U.S. FDA, 2010) and will substitute for currently marketed L-leucine products. L-Leucine is proposed for use as an ingredient in milk and non-milk based meal replacements, sports and isotonic beverages, vitamin enhanced waters, and meal replacement bars, at levels of 0.5-3.0 g/serving.

Consequently, INNOBIO<sup>®</sup> notes that its uses will not result in any exposure beyond what was previously estimated. Based on previous analysis of NHANES 2005-2006 (CDC, 2006; GRN 308, U.S. FDA, 2010), the proposed food uses would result in an EDI for users (mean of 1.9 g/person/day or 28 mg/kg body weight/day and 90th percentile intake of 4.1 g/person/day or 64 mg/kg body weight/day) at levels significantly below those associated with any side effects. In addition, these estimates are highly optimistic since it is not likely that L-leucine will be used at maximum levels for all food categories under the intended uses. Also, food wastes should be considered. Overall, intended use will result in EDIs at levels significantly below those associated with any potential side effects.

## **IV. BASIS FOR GRAS DETERMINATION**

### **IV.A. Current Regulatory Status**

L-Leucine, free, hydrated, or anhydrous or as the hydrochloride, sodium or potassium salts, is permitted for use as a special dietary and nutritional additive to significantly improve the biological quality of the total protein in a food containing naturally occurring primarily intact protein that is considered a significant dietary protein source (21 CFR 172.320) (U.S. FDA, 2009). As a food additive, L-leucine, should meet the specifications established by the FCC, NAS/NRC, and the amount of L-leucine added for nutritive purposes, plus the amount naturally present in free and combined (as protein) form should not exceed 8.8% by weight of the total protein of the finished food. L-Leucine also may be used as a lubricant in the manufacture of aspartame or neotame tablets for sweetening hot beverages at levels not to exceed 3.5% of the

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tablet weight (21 CFR 172.804; 21 CFR 172.829; U.S. FDA, 2009). Additionally, L-leucine is deemed to be GRAS by the FEMA under specific conditions of use.

In Europe, L-leucine has E number E641 and is classified as a flavor enhancer. E numbers are codes for substances which can be used as food additives for use within the European Union (EU) and they are commonly found on food labels throughout the EU.

### *Corynebacterium glutamicum*

The FDA indicated that it had no questions in response to a GRAS Notice for lysine monohydrochloride (GRN 000414), produced using fermentation of sugars and molasses with *Corynebacterium glutamicum* (U.S. FDA, 2012).

## **IV.B. Intended Technical Effects**

L-leucine can be used as a food ingredient at concentrations consistent with cGMP.

## **IV.C. Review of Safety Data**

Our review includes the safety data listed in GRN 308 (U.S. FDA, 2010). Since the FDA's last review of L-leucine in 2009-2010 (GRN308; U.S. FDA, 2010), no literature contradicting the agency's prior decision has been published.

### **IV.C.1. Metabolic Fate of L-Leucine**

L-leucine absorbed from the gastrointestinal tract into the enterocytes as either free amino acids or as constituents of peptides. Following absorption into enterocytes, L-leucine is transported to the liver, where a portion of the L-leucine is used for protein synthesis (Physicians' Desk Reference for Nutritional Supplements [PDRNS], 2008). The remaining L-leucine is distributed via the systemic circulation to various tissues of the body (PDRNS, 2008).

In humans, the majority of L-leucine metabolism occurs in the skeletal muscle (Rosenthal, 1974; PDRNS, 2008). L-Leucine is degraded by deamination (removal of the amino group). The amino portions are captured as urea via the Krebs-Henseleit cycle in the liver and urea is excreted in urine by the kidneys (IOM, 2005). The nitrogen-free portion (deaminated amino acid) enters the metabolic pathway and forms acetyl CoA, which can be used as a source of energy via the pathways of oxidative metabolism.

L-Leucine undergoes reversible transamination, catalyzed by branched-chain aminotransferase (BCAT), to yield alpha-ketoisocaproate (KIC; IOM, 2005). This product then undergoes an irreversible oxidative decarboxylation by branched-chain alpha-ketoacid dehydrogenase (BCKDH) to produce isovaleryl CoA, an acyl CoA derivative (Harper et al., 1984; IOM, 2005). This undergoes further metabolism through several steps, eventually resulting in the production of acetoacetate and acetyl CoA, which can be used as a source of energy *via* the pathways of oxidative metabolism (Harper et al., 1984; IOM, 2005). BCKDH kinase is inhibited by KIC and other branched-chain keto acids (i.e., the substrates for BCKDH), allowing for enhanced BCAA metabolism when they are present in excess amounts and decreased metabolism when they need to be conserved.

BCAA and other large neutral amino acids (LNAAs) compete for transport across membranes including the blood-brain barrier; Infusion of L-leucine into the rat brain has been shown to decrease food intake and body weight via activation of the mammalian target of rapamycin (mTOR) pathway (Cota et al., 2006). However, there is no evidence in animal studies of neurotoxic effects following long-term L-leucine consumption, nor have there been reports of neurotoxicity in humans receiving oral L-leucine or BCAA supplementation.

BCAAT exists in at least three different subtypes, and its tissue and cellular distribution varies across species. Differences between rats and humans in this regard raise the possibility that, to the extent that adverse biological effects of the BCAA are dependent upon metabolism, rodent data may not be completely predictive of human responses (Harper et al., 1984). It should be noted, however, that in most of the animal studies, it is not entirely clear that these various enzyme activities are critical determinants of the effects seen (IOM, 2005).

### **IV.C.2. Mutagenicity and Genotoxicity Studies of L-Leucine**

As discussed in GRN 308, L-leucine at a concentration of 2 mM (234 µg/mL) was not mutagenic in *E. coli* strains *uvr6*, *uvrB umu C*, and *uvrB LexA* in the absence of metabolic activation (Sargentini and Smith, 1986). In another study, L-leucine at concentrations of 10, 50, or 100 mg/mL produced slight, yet significant increases in sister chromatid exchanges (SCEs) in human lymphocytes compared to a control group (Xing and Na, 1996). The authors suggested that the elevated frequencies of SCEs were considered to be metabolic rather than genotoxic responses, since the effect was not dose-dependent. Since the last FDA's review of L-leucine in 2009-2010 (GRN308; U.S. FDA, 2010), there is no literature on this topic has been published.

### **IV.C.3. Animal Toxicity Studies of L-Leucine**

Our review includes the toxicity studies reported in GRN 308, such as a 13 week rat toxicity study of Tsubuku et al. (2004), a reproductive and developmental toxicity study of Mawatari et al. (2004), and other studies providing safety-related endpoints.

A recent animal toxicity study (Imamura et al., 2013) confirmed the previous finding that the NOAEL for the L-leucine is 5.0% or higher for both genders (Tsubuku et al., 2004). This was the highest level fed, which is equivalent to 3,333 mg/kg body weight/day for males and 3,835 mg/kg body weight/day for females (GRN 308; U.S. FDA, 2010). It is also supported by a rat study by Imamura et al. (2013) in which L-leucine intake up to 8% (or 5,300 mg leucine/kg body weight/day) for 1 week had no adverse effects except when animals were fed a low-protein diet. Based on the maximum metabolic limit or oxidation of L-leucine, Sakai et al. (2004) identified the UL as 10% of diet or 8,900 mg/kg body weight/day in male Fischer rats.

For reproductive and developmental toxicity, the previously found NOAEL of 1,000 mg/kg body weight/day (Mawatari et al., 2004) is still valid. The study of Chen et al. (2012) found that the NOAEL for was 1.5% in drinking water, the highest level tested. The studies published since 2010 are summarized in Table 5.

### Subchronic toxicity studies

Tsubuku et al. (2004) conducted a 13-week long dose-response study evaluating the toxicological and behavioral effects of L-leucine with male and female Sprague-Dawley rats. The amino acids were incorporated into a standard diet at doses equal to 1.25%, 2.5%, and 5.0% (w/w). A control group of rats received a standard diet. All diets were administered ad libitum for 13 consecutive weeks. To examine stability of any potential effects, the administration period was followed by a 5-week recovery period, during which only the standard diet was provided to all animals. No significant, dose-related effects on body weight were found in rats fed a leucine-supplemented diet. No significant effects on organ weights, hematology, blood chemistry, urinalysis, and histopathology were recorded. The authors suggested the NOAEL for L-leucine to be 5.0% in diet for both genders (males,  $3,333 \pm 101$  mg/kg body weight/day; females,  $3,835 \pm 257$  mg/kg body weight/day).

### Developmental and reproductive toxicity studies

Chen et al. (2012) investigated the effect of dietary L-leucine supplementation on hypothalamic rapamycin, thus food intake, and metabolic disorders in the offspring of obese dams. Obesity was induced in Sprague-Dawley female rats by high-fat diet for 5 weeks before mating, throughout gestation and lactation. At weaning (20 days), pups from lean mothers were fed a chow diet (control) for 10 weeks with free access to water. Pups from high fat diet (HFD)-fed mothers were divided into two dietary groups, chow and HFD, for 10 weeks. Within each dietary cohort, half of the pups were supplied with L-leucine via drinking water (1.5% w/w) versus water control. Those from chow-fed mothers were fed chow and water. This yielded five experimental groups, chow control, HFD-chow, HFD-chow+leucine, HFD-HFD and HFD-HFD+leucine (n = 12). Maternal obesity led to increased adiposity in chow-fed offspring. At 20 days, offspring from obese dams were 42% heavier than those from lean dams. At 13 weeks, they consumed 7% more calories, although this was not statistically significant. Postweaning HFD consumption exaggerated hyperglycemia, hyperinsulinemia, and hyperlipidemia. At 13 weeks, HFD-fed rats had a 60% increase in blood glucose levels and a three-fold increase in plasma insulin levels compared to their chow-fed littermates. High-fat diet consumption also doubled the plasma and liver triglyceride (TG) levels, and lead to a 13% increase in plasma non-esterified fatty acid (NEFA) levels ( $P < 0.05$ ). Daily L-leucine intake was almost doubled in leucine-treated rats compared to littermates on the same diet. However, L-leucine supplementation did not affect appetite regulation, 24 h energy intake, or body weight. An interaction effect of L-leucine and HFD led to significantly lower blood glucose levels during the entire intraperitoneal glucose tolerance test (IPGTT), as well as decreased area under the curve (AUC) in the HFD-HFD+leucine group compared to the HFD-HFD group. Fasting plasma glucose and insulin concentrations were not influenced by L-leucine supplementation regardless of diet. L-leucine supplementation also led to a reduction of plasma TG (by 16-28%) in offspring regardless of diet in HFD fed rats. Hypothalamic p-mTOR was not altered by maternal obesity, but was significantly increased by leucine treatment. Results suggest that L-leucine may play a role in the management of metabolic disorders due to the ability to improve peripheral glucose and lipid metabolism independent of appetite and weight regulation in offspring from obese dams. The NOAEL for female reproductive function and embryo-fetal development was concluded to be 1.5% in drinking water, the highest dose assessed in the study. These levels may correspond to 782 mg/kg body weight/day for males and 836 mg/kg body weight/day for female rats.

For reproductive and developmental toxicity, the previously found NOAEL of 1,000 mg/kg body weight/day (Mawatari et al., 2004) is still valid. In this study, 11-12-week old dams were orally administered an aqueous solution of L-leucine at doses of 300 or 1,000 mg/kg body weight/day on gestational days 7-17. The body weight and feed intake were measured during the course of pregnancy, and detailed placental, as well as fetal examinations were conducted immediately after Caesarean section on day 20 of pregnancy. No deaths and no changes in the general condition were observed in either of the groups administered the test substances or in the controls. Results suggested that L-leucine, administered orally during organogenesis at doses up to 1,000 mg/kg body weight/day, did not affect the outcome of pregnancy and did not cause fetotoxicity in rats. Overall, the NOAEL for female reproductive function and embryo-fetal development was concluded to be 1,000 mg/kg body weight/day, the highest dose assessed (Chen et al., 2012; Mawatari et al., 2004).

### Studies determining UL

In determining UL of L-leucine, Sakai et al. (2004) identified “metabolic limits” by measuring CO<sub>2</sub> arising from amino acid excess. Male Fisher rats were fed a commercially produced nonpurified diet for 1 week, then the modified American Society of Nutrition (ASN)-93G diet for 1 week, then switched to experimental diets in which L-leucine was added in graded levels of 0, 2.5, 5, 7.5, 10, 15, 20, or 30% in the modified ASN-93G diet in place of cornstarch. They were trained to complete each meal within 9 hours for 10–14 days. For the labeled diets, [U-<sup>13</sup>C]-L-leucine was mixed into the additional L-leucine at the constant ratio of 5% of total L-leucine. The labeled diets (equal amounts of food intakes of the day before) were given to the rats to collect the expired CO<sub>2</sub> for the subsequent 24 h. Uniformly <sup>13</sup>C labeled L-leucine was used as tracer in diets with added L-leucine fed to rats, <sup>13</sup>CO<sub>2</sub> arising from its metabolism was collected over 24 h, and the fraction of the ingested L-leucine that was exhaled as CO<sub>2</sub> was calculated. The maximum limit to oxidize excess L-leucine was reached at 10% of dietary intake or 8,900 mg/kg body weight/day and the oxidation achieved a plateau. This inflection point was identified as the UL of L-leucine intake in rats.

Imamura et al. (2013) conducted a rat Sprague-Dawley study to identify reliable gene biomarkers for the adverse effects of excessive L-leucine by DNA microarray. It has long been known that the adverse effects of excessive amino acid intake depend on dietary protein levels. Male rats were divided into 12 groups (n=6) and fed for 1 week a diet containing low (6%), moderate (12%), or high (40%) protein. Different levels of L-leucine (0, 2, 4, and 8%) were added to the diets. Endpoints were food intake and DNA transcriptomic analysis. Consumption of diets containing more than 4% L-leucine in 6% protein diet resulted in growth retardation (body weight gain: 0% vs. 4% vs. 8%= +25 vs. 0 vs. -40 g) and reduced liver weight (liver weight/body weight, %: 0% vs. 4% vs. 8%=3.4 vs. 3 vs. 2.5), whereas the administration of the same dose of L-leucine with 12% or 40% protein diet did not affect them. In other words, L-leucine intake up to 8% had no adverse effects when protein intake was adequate. The gene-marker panel and growth pattern suggested that for male rats dietary L-leucine supplementation of 2% is the NOAEL dose in low-protein (6%) diets. In an adequate protein diet (over 12% in diet), the NOAEL appears to be 8% L-leucine in diet which may correspond to 5,300 mg/kg body weight/day.

Table 5. Animal Toxicity Studies of L-Leucine Published Since 2010

Animal, species (N)	Dose	Duration	Outcomes tested	Outcomes	Reference
Rat, Sprague-Dawley (72)	0, 2, 4, and 8% L-leucine in a diet containing low (6%), moderate (12%) or high (40%) protein	1 week	Food intake and DNA transcriptomic analysis	NOAEL in low-protein (6%) diets-2% leucine. In an adequate protein diet (over 12% in diet), the NOAEL appears to be 8% L-leucine in diet.	Imamura et al., 2013
Rat, Sprague-Dawley (Dams: Not Specified, Pups: 12/diet group)	<u>Dams</u> (2 diet groups): 1) Control group -standard rodent chow: 11 kJ/g, 4% fat, 14% protein, L-leucine 1.7% 2) Test group-pellet HFD: 20 kJ/g, 23% fat, 19.4% protein, L-leucine 1.7% <u>Pups</u> (5 diet groups): 1) Chow-Chow 2) HFD-chow 3)HFD-chow+leucine 4) HFD-HFD 5) HFD-HFD+leucine Note: L-leucine was supplied via drinking water (1.5% w/w)	Dams: 5 weeks prior to mating Pups:10 weeks	Energy intake, IPGTT, dam fat mass, plasma and liver TG, plasma NEFA, leptin, insulin, total RNA, phosphorylated - and total signal transducer and activation of transcription including mTOR	Daily L-leucine intake was almost doubled in leucine-treated rats compared to littermates on the same diet. However, L-leucine supplementation did not affect appetite regulation, 24 h energy intake, body weight, fasting plasma glucose and insulin conc. An interaction effect of L-leucine and HFD led to significantly lower blood glucose levels during the IPGTT and reduction of plasma TG (by 16-28%) in offspring regardless of diet in HFD fed rats. Hypothalamic p-mTOR was not altered by maternal obesity, but was significantly increased by L-leucine treatment. The NOAEL; 1.5% in drinking water (782 mg/kg body weight/day for males and 836 mg/kg body weight/day for female rats), the highest dose assessed.	Chen et al., 2012

HFD=high fat diet; IPGTT=intraperitoneal glucose tolerance test; mTOR=mammalian target of rapamycin; NEFA = non-esterified fatty acid; RNA = ribonucleic acid; TG = triglycerides.

### Carcinogenicity study

Three carcinogenicity studies reported that rats exposed to low doses of N-butyl-N (4-hydroxybutyl) nitrosamine (BHBN), an initiator of urinary bladder carcinoma, were given diets supplemented with 2 or 4% (w/w) L-leucine (corresponding to approximately 1,000 and 2,000 mg/kg body weight/day, respectively; U.S. FDA, 1993) for 40 or 60 weeks (Kakizoe et al., 1983; Nishio et al., 1986; Xie et al., 2012). Increased incidence and number of carcinomas were reported in the group treated with BHBN plus L-leucine compared to the group treated with BHBN alone. However, no lesions in the urinary bladder were reported in the group receiving diets supplemented with L-leucine without BHBN (IOM, 2005).

For example, Nishio et al. (1986) evaluated the effect of L-leucine supplementation for 40- to 60-weeks on bladder cancer in rats. Two experiments were conducted, one involving 155 F344 rats to examine supplementation with 2.0% L-leucine on bladder carcinogenesis over 40 weeks. The group receiving BHBN plus 2.0% L-leucine also had a greater carcinoma incidence and number compared to the BHBN (control) group, but this was not significant. The second experiment involved 217 male F344 rats and examined the dose dependency of L-leucine over 60 weeks in bladder carcinogenesis. The incidences and numbers of carcinomas were significantly higher in groups treated with BHBN and amino acids (2.0 or 4.0% each of L-leucine and L-isoleucine) than in the group treated with BHBN alone, and the amino acids exhibited dose-dependent effects.

The study of Xie et al. (2012) investigated effects of L-leucine on rat bladder carcinogenesis using American Society for Nutrition (ASN)-93G basal diet. In Experiment 1, BHBN was used as an initiator of bladder carcinogenesis. In the ASN-93G diet groups, a significantly higher incidence and multiplicity of bladder tumors, accompanied by decreased final body weight, was observed in the L-leucine-supplemented group. Urinary pH values were not affected by L-leucine supplementation. In Experiment 2, the amino acid was administered in the basal diets for 2 weeks without initiator. No pathological lesions were observed in the bladder urothelium in any of the groups, and no significant differences in urinary pH values, microcrystals or aggregates were observed between the amino acid supplemented groups and their respective control groups. The results indicated that long term supplementation of L-leucine was not carcinogenic in normal rats with no induced bladder cancer (IOM, 2005). It should be also noted that tumor promotion studies have not been designed or validated for the purposes of hazard assessment (Kraus et al., 1995).

### Other studies providing safety-related endpoints

Results from additional rodent studies on L-leucine, which were not conducted primarily to assess safety but included measurements of some safety related endpoints, corroborate the safety information provided from animal toxicity studies. In these studies, L-leucine was consumed in the diet or drinking water at levels of up to 5% in diet (Campos-Ferraz et al., 2013; Donato et al., 2006; Li et al., 2013; Lynch et al., 2002; Pereira et al., 2014; Sugawara et al., 2007; Torres-Leal et al., 2011; Witham et al., 2013; Zhang et al., 2007).

Witham et al. (2013) studied the effect of L-leucine on cardiac injury and survival post-myocardial infarction (MI) in 11-12 week old male C57BL/6 mice subjected to either an

experimental myocardial infarction (MI) or sham procedure, and provided regular chow (1.5% L-leucine) or a high L-leucine diet (5% L-leucine), and followed for 3.5 or 28 days. All mice were studied by echocardiography and cardiac catheterization, and all hearts were collected for histologic measurements of hypertrophy, fibrosis, and apoptosis. Inflammation, hypertrophic gene expression, signal transduction, and glucose, palmitate and L-leucine metabolism were also measured in the hearts of mice followed for 3.5 days. Results suggested that dietary L-leucine increased cardiac hypertrophic signaling, attenuated myocardial fibrosis and apoptosis, and improved cardiac performance post-MI. Moreover, a significant survival benefit was observed with L-leucine consumption post-MI. No adverse effects of L-leucine supplementation up to 5% in diet (highest dose tested) were reported on measured outcomes.

The study of Torres-Leal et al. (2011) showed that L-leucine supplementation was effective in increasing adiponectin levels and in reducing total cholesterol (TC) concentration although L-leucine supplementation had no beneficial effects on improving insulin sensitivity or adiposity in previously obese rats. The aim of this study was to determine the effect of chronic L-leucine supplementation alone or combined with endurance training as potential approaches for reversing the insulin resistance and obesity induced by a HFD in rats. In this study, 47 rats were randomly divided into two groups: control diet-low fat (n = 10) or HFD (n = 37). After 15 weeks on HFD, all rats received the control low fat diet and were randomly divided according to treatment: control (14% casein), 5% L-leucine supplementation (in addition to 14% casein), endurance training, and 5% L-leucine + endurance training (n = 7-8 rats per group). At the end of the sixth week of treatment, there was no significant difference in body weight between the groups. L-leucine supplementation increased serum adiponectin (P = 0.021) levels and reduced serum TC concentration (P = 0.042). No adverse effects of L-leucine supplementation on measured outcomes were reported.

Li et al. (2013) investigated the effect of chronic L-leucine supplementation on insulin sensitivity and the associated mechanisms in rats on HFD. Male Sprague-Dawley rats were fed either normal chow diet or HFD supplemented with 0, 1.5, 3.0, or 4.5% leucine for 24 weeks. Chronic leucine supplementation increased insulin sensitivity together with increased body weight in rats on HFD, but had no effect on insulin sensitivity in rats on normal chow diet. The increased insulin sensitivity by leucine supplementation was not associated with altered ectopic fat accumulation in liver or muscle, plasma levels of lipids and cytokines, but was associated with reduced oxidative stress and improved insulin signaling, insulin-stimulated protein kinase B, and mTOR phosphorylation in liver, skeletal muscle, and adipose tissue of rats on HFD rats. The data indicate that leucine supplementation prevented HFD-induced insulin resistance in insulin-target tissues. No adverse effects on L-leucine were reported.

Pereira et al. (2014) reported that leucine supplementation (1,350 mg/kg body weight/day for 10 days) improved skeletal muscle regeneration process, in particular myofiber size and strength recovery after cryolesion in rats. No adverse effects on L-leucine were reported.

In the study of Campos-Ferraz et al. (2013), all male Wistar rats were submitted to swimming exercise for 6 weeks and supplemented with either the mixture of BCAAs, leucine, or placebo during the last week of training (166 mg of the corresponding amino acids/kg body weight/day diluted in 2 mL distilled water) for 7 days. At week 7 of the protocol, the rats were submitted to

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an intermittent, progressive swimming test until exhaustion and sacrificed. Muscle gastrocnemius and liver were depicted to determine total glycogen, tricarboxylic acid cycle (TCA) intermediates, and enzymatic activities. No adverse effects of L-leucine were reported on measured outcomes.

Lynch et al. (2002) examined the effects of 12 day supplementation of chronic L-leucine and L-leucine mimetics (norleucine) via drinking water (114 mM L-leucine or norleucine) on protein synthesis in adipose tissue, kidney, heart, liver, and skeletal muscle from the ad libitum-fed rats. The control diet consumed L-leucine at the level of 2,800-3,000 mg/kg body weight/day, while the two supplement groups consumed the doubled amount of L-leucine plus norleucine from water and food (~5,600-6,000 mg/kg body weight/day) within a day and was maintained at this level over the entire protocol period. Results of the study suggested that provision of L-leucine or norleucine supplementation via drinking water stimulated postprandial protein synthesis in adipose tissue, skeletal muscle, and liver without notable adaptive changes in signaling proteins or metabolic enzymes.

In the study of Donato et al. (2006), adult male Wistar rats underwent 50% food restriction for 6 weeks. The control group received the ASN-93G diet and the L-leucine group received the same diet supplemented with 5.91 g L-leucine/kg ration. Low-dose L-leucine supplementation increased body fat loss and improves liver protein status and the capacity of muscle protein synthesis without changing body weight in rats underwent food restriction.

Sugawara et al. (2007) reported that supplementation with L-leucine at 1.5% to a protein-deficient diet for a week suppressed myofibrillar protein degradation measured by the rate of release of 3-methylhistidine (MeHis) from isolated extensor digitorum longus (EDL) muscle by approximately 30% in rats. When rats were fed a 5% casein diet, the gastrocnemius muscle weight decreased and MeHis released from the EDL muscle increased compared to those fed a 20% casein diet. However, feeding of a 5% casein diet supplemented with L-leucine (1.15%) reduced muscle weight loss and MeHis release.

Zhang et al. (2007) reported increasing dietary L-leucine intake reduced diet-induced obesity and improved glucose and cholesterol metabolism in male C57BL/6J mice. L-leucine was supplemented via drinking water made of 1.5% (w/v) for 14 weeks. Leucine-supplemented mice ingested an additional 55 mg L-leucine daily via water consumption, which nearly doubled the total daily L-leucine intake from food (56 mg from chow; 63 mg from HFD). Both control and experimental mice had free access to either rodent chow or a HFD. While it produced no major metabolic effects in chow-fed mice, increasing L-leucine intake resulted in up to 32% reduction of weight gain and a 25% decrease in adiposity in HFD-fed mice, while food intake was not decreased. Increasing L-leucine intake also prevented HFD-induced hyperglycemia and decreased plasma concentrations of TC and low density lipoprotein cholesterol (LDL-C) by 27% and 53%, respectively. The authors concluded that doubling dietary L-leucine intake on a chronic basis produces a net health benefit that includes reduction of diet-induced weight gain, hyperglycemia, and hypercholesterolemia in mice on a HFD.

#### **IV.D Human Studies**

The GRN 308 reported that studies examining oral L-leucine supplementation in humans did not find adverse effects of L-leucine (Swendseid et al., 1965; Mero et al., 1997, 2009; Pitkanen et al., 2003; Scarna et al., 2003; Crowe et al., 2006; Verhoeven et al., 2009). These studies reported that doses up to 24 g/day were well tolerated. In addition, recent human studies examining oral L-leucine supplementation on performance during exercise training or other outcomes also reported no adverse effects of L-leucine (Bjorkman et al., 2011; Casperson et al., 2012; Ispoglou et al., 2011; Kirby et al., 2012; Leenders et al., 2011; Walker et al., 2010; Zemel et al., 2012). Doses tested were up to 12 g/day and the duration of the studies were up to 6 months. The majority of the studies did not include assessment of safety-related endpoints, but rather focused on the effects of acute and prolonged dietary intake of L-leucine in individuals undergoing exercise training; however, the information in the human studies corroborates the safety information provided from animal studies.

#### Studies determining UL

The IOM (2005) has no established UL of L-leucine. Recently, the ICAAS (Cynober et al., 2012) has proposed an upper limit of safe intake for L-leucine for healthy adults as 0.53 g/kg body weight/day, which corresponds to 37.1 g/day in a 70 kg adult. It is based on 3 human studies (Elango et al., 2012b; Pencharz et al., 2012; Pencharz and Russell, 2012) and a rat study of Imamura et al. (2013). These human studies described the effects of acute, 1-day ingestion of graded doses of L-leucine on a variety of metabolic variables. This approach was used rather than chronic ingestion of high doses of L-leucine. In these studies, 5 young (25–35 years), healthy men received graded stepwise increases in intakes of 50, 150, 250, 500, 750, 1000, and 1250 mg/kg body weight/day; 7 dosages) which corresponded to the EAR and the EAR $\times$ 3,  $\times$ 5,  $\times$ 10,  $\times$ 15,  $\times$ 20, and  $\times$ 25 in a total of 29 studies. The endpoints were L-leucine oxidation and other surrogate markers such as plasma glucose, alanine aminotransferase, and ammonia to monitor for adverse effects. The UL of L-leucine was identified by the measurement of plasma and urinary biochemical variables and changes in L-leucine oxidation by using L-[1-<sup>13</sup>C]-leucine. With L-leucine intakes >500 mg/kg body weight/day, significant increases in blood ammonia concentrations above normal values, plasma L-leucine concentrations, and urinary L-leucine excretion were observed. A plateau in L-[1-<sup>13</sup>C]-leucine expressed as label tracer oxidation in breath was observed after 500 mg/kg body weight/day, no clear plateau was observed in L-leucine oxidation, and  $\alpha$ -KIC oxidation appeared to plateau after 750 mg/kg body weight/day. In other words, the metabolic limit to oxidize L-leucine was between 550 and 700 mg/kg body weight/day, after which plasma L-leucine progressively increased and plasma ammonia also increased in response to L-leucine intakes. The increase in ammonia was not the result of liver cell damage as liver enzymes levels did not change. No gut intolerance was seen. Blood glucose fell progressively but remained within normal values without any changes in plasma insulin. The authors estimated the safe upper limit of L-leucine intake as 550 mg/kg body weight/day in humans.

It is also supported by a rat study of Imamura et al. (2013) in which leucine intake up to 8% of diet had no adverse effects except when animals were fed a low-protein diet. This intake corresponds to 5.3 g leucine/kg body weight/day. Considering that protein and energy requirements in rats are 10 times those of humans and considering normal healthy people have

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moderate protein intakes and those practicing exercise or sport have generally high protein intakes, authors set UL of L-leucine in humans at 530 mg/kg body weight/day in humans.

The 90<sup>th</sup> percentile intakes of users under the intended use (64 mg/kg body weight/day) are far below this proposed UL. Overall, the body of evidence on oral administration of L-leucine in humans indicates that it can be consumed in considerable amounts without adverse effects.

### Efficacy studies

Several studies examined oral L-leucine supplementation on performance during exercise training or other outcomes in humans (Bjorkman et al., 2011; Casperson et al., 2012; Crowe et al., 2006; Ispoglou et al., 2011; Kirby et al., 2012; Leenders et al., 2011; Mero et al., 1997, 2009; Pitkanen et al., 2003; Swendseid et al., 1965; Verhoeven et al., 2009; Walker et al., 2010; Zemel et al., 2012). Studies published since 2010 are summarized in Table 6. Although these studies did not include assessment of safety-related endpoints, the information in the human studies corroborates the safety information provided from animal studies. Daily supplementation doses up to 12 g/day were well tolerated in health subjects and subjects with type 2 diabetes.

Bjorkman et al. (2011) reported that a low intensity exercise program combined with post-exercise milk protein supplementation is feasible and seemed effective in improving muscle mass and functions of older persons with inflammatory disease. In this study, 47 participants with polymyalgia rheumatica (mean age of 69.5 years) received supplements consisted of either a regular casein based dairy product (3.3 % protein in diet; whey:casein ratio=20:80) or a whey protein-enriched dairy product with a higher L-leucine content (providing additional 2.3 g/day leucine and 15 g/day protein; 7.0% protein in diet; whey:casein ratio=80:20) for 8 weeks after exercise. The authors reported no severe adverse events related to leucine supplementation. However, some gastrointestinal complaints such as early satiety, flatulence, nausea, and diarrhea were reported. These complaints tended to be more common with the test supplement compared to the control supplement (44.7% vs. 32.6%, P=0.180).

Ispoglou et al. (2011) investigated the effects of daily oral L-leucine ingestion on strength, bone mineral-free lean tissue mass, and fat mass of free-living humans during a 12 week resistance-training program. Twenty-six untrained men (n=13 per group) ingested either 4 g/day of L-leucine or a corresponding amount of lactose (placebo group). Over the 12 week period the L-leucine group had significantly greater percentage mean gains compared to the placebo group in 5 out of 8 exercise tests and total strength (P<0.05). Significant differences did not exist between groups in either total percentage lean tissue mass gains or total percentage fat mass losses. No adverse events were reported.

Casperson et al. (2012) supplemented 12 g leucine/day (4 g/meal; 3 meals/day; days 2-14) for 2 weeks to determine the effect on mixed muscle protein synthesis, body composition, and markers of nutrient signaling including mTOR in older adults (n=8). Two weeks of L-leucine supplementation increased post-absorptive muscle protein synthesis by 15% (Day 1:  $0.063 \pm 0.004$  vs. Day 15:  $0.074 \pm 0.007\%/h$ ;  $p = 0.004$ ) and nutrient anabolic signaling as evidenced by increased phosphorylation of mTOR and other markers of nutrient signaling. No change in fat free mass was observed. No adverse effects on measured outcomes were reported.

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A six month study by Leenders et al. (2011) assessed the effect of 7.5 g/day L-leucine (2.5g/meal x 3 meals/day) compared to a placebo (7.5 g/d wheat flour) in 60 elderly men (30 per group) with type 2 diabetes. Body composition, muscle fiber characteristics, muscle strength, glucose homeostasis, and basal plasma amino acid and lipid concentrations were assessed prior to, during, and after the intervention. No group differences were noted in lean tissue mass, body fat percentage, muscle strength, muscle fiber type characteristics, blood glycosylated hemoglobin, oral glucose insulin sensitivity, and plasma lipid concentrations. The authors did not report any adverse effects related to L-leucine consumption.

Kirby et al. (2012) determined the short term effects of L-leucine supplementation on indices of muscle damage following eccentric-based resistance exercise in 27 untrained males randomized to one of 3 groups: L-leucine (+ 3 g Splenda), placebo (3 g Splenda), and control (no treatment). L-leucine at a dose of 250 mg/kg body mass or placebo was consumed 30 minutes before, during and immediately after exercise and the morning of each recovery day following exercise (24, 48, 72 and 96 h). The control group did not perform the resistance exercise protocol nor ingest any form of supplement. High-dose L-leucine supplementation was unable to attenuate the increase in biochemical markers of muscle damage that follows eccentric-based resistance exercise, however, it may aid in the maintenance of isometric force output. Muscle soreness increased across all time points for both the L-leucine and placebo group, and the L-leucine group experienced a significantly higher increase in mean post-exercise muscle soreness of the lower body. The authors noted their findings are in conflict with previous investigations that either showed no effect of leucine or BCAAs on muscle soreness (Stock et al. 2010), or an attenuation in the severity of the soreness (Jackman et al.2010; Shimomura et al. 2006). Overall, no consistent effects of L-leucine on muscle soreness were noted from the literature.

Walker et al. (2010) investigated the effects of supplementation of 19.7 g/day whey-protein and 6.2 g/day L-leucine on physical and cognitive performance and body composition in 30 moderately fit men. The 8 weeks of supplemental whey protein with L-leucine resulted in mild increases in muscle strength and lean body mass (due to increases in bench press [significant] and push up [non-significant] performance), but did not promote increases in endurance performance or cognitive performance. No adverse effects of L-leucine were observed.

In a study of Zemel et al. (2012), a nutraceutical containing 2.25 g L-leucine and 30 mg pyridoxine (vitamin B6) was tested in 20 overweight or obese participants. Participants received the blend containing 2.25 g L-leucine or placebo (each dose = 750 mg L-leucine and 10 mg pyridoxine, 3 times/day) for 4 weeks without energy restriction. The blend resulted in increased fat oxidation by 33.6 g/day ( $p < 0.04$ ), decreased respiratory quotient, improved homeostatic insulin resistance (HOMA-IR), and reduced oxidative and inflammatory biomarkers (plasma concentrations of malondialdehyde [MDA], 8-isoprostane-F<sub>2α</sub>, tumor necrosis factor alpha [TNF $\alpha$ ], and C reactive protein [CRP]). No adverse effects of L-leucine were observed.

Overall, no significant adverse effects of L-leucine were reported from recent human clinical studies when daily doses were up to 12 g.

Table 6. Human Studies of L-Leucine Published Since 2010

Subjects	Daily Dose	Duration/Design	Measured Outcomes	Results	Reference
26 Untrained Men (Mean age ~28; n=13 group)	4 g leucine	12 weeks, P	Body composition (lean tissue mass), and strengths on each exercise	No adverse effects on measured outcomes.	Ispoglou et al., 2011
8 Healthy, sedentary older adults (Mean age not given)	4 g/meal 3x/day=12 g	2 weeks, X	Body composition, and protein synthesis	No adverse effects on measured outcomes.	Casperson et al., 2012
60 Elderly males with T2DM (Mean Age 70; n=30 group)	2.5 g/meal x 3 meals= 7.5g	6 months, P	Body composition, muscle fiber characteristics, muscle strength, glucose homeostasis, and blood amino acids and lipid concentrations	No adverse effects on measured outcomes..	Leenders et al., 2011
30 moderately fit males (Mean age 26.9; Protein-18, Placebo-12)	19.7 g of whey protein and 6.2 g of leucine	8 weeks, P	exercise performance characteristics, body composition, and cognitive performance	No adverse effects on measured outcomes.	Walker et al., 2010
20 overweight or obese subjects (Mean age 29; n=10 group)	2.25 g L-leucine and 30 mg pyridoxine (test) or 750 mg L-leucine and 10 mg pyridoxine (control)	4 weeks, P	Resting metabolic rate, and plasma MDA, plasma 8-isoprostane, IL-6, adiponectin, TNF- $\alpha$ , and CRP	No adverse effects on measured outcomes.	Zemel et al., 2012

CRP=C reactive protein; IL=interleukin; MDA=malondialdehyde; P=parallel; T2DM= type 2 diabetes mellitus; TNF $\alpha$  = Tumor necrosis factor alpha; X=crossover.

Table 6. Human Studies of L-Leucine Published Since 2010, continued

Subjects	Daily Dose	Duration/ Design	Measured Outcomes	Results	Reference
27 Untrained males (Mean Age 21; Leucine n=10, Placebo n=9, Control n=8)	250 mg/kg body weight	Up to 96 h; L-leucine intervention on recovery following exercise, P	Peak force during squat, Jump height, creatine kinase, myoglobin, and subjective feeling	Muscle soreness for the L-leucine and placebo groups significantly increased following exercise at the 24, 48, 72 and 96 h. The L-leucine group had significantly higher mean muscle soreness across all post-exercise time points. No other adverse effects were reported.	Kirby et al., 2012
47 Finnish elderly suffering from polymyalgia rheumatic (Mean Age 69.5)	Test -whey protein enriched (additional 2.3 g/day leucine) vs. casein enriched diet (control)	8 weeks, X	Body composition, grip strength, jump performance, chair stand test, and walking speed.	No severe adverse events occurred; however, test group tended to show higher incidence of some gastrointestinal complaints such as early satiety, flatulence, nausea, and diarrhea (NS). Overall, no adverse effects of L-leucine were reported on measured outcomes.	Bjorkman et al., 2011
5 Healthy young adults (20-35y)	Stepwise increments of L-leucine at 50, 150, 250, 500, 750, 1000, and 1250 mg/kg to determine tolerable upper intake level	Graded, stepwise increments of L-leucine over 29 studies, X	Body weight, blood, urine, and breath gasses	A UL for L-leucine in healthy adult men was suggested at 500 mg/kg/d or ~35 g/d based on the observed significant increases in blood ammonia concentrations above normal values, plasma L-leucine concentrations, and urinary L-leucine excretion observed with L-leucine intakes >500 mg/kg/d. In addition, a plateau in L-[1- <sup>13</sup> C]-leucine was observed after 500 mg/kg/d, no clear plateau was observed in L-leucine oxidation, and a plateau in KIC oxidation after 750 mg/kg/d.	Elango et al., 2012b; Pencharz et al., 2012; Pencharz and Rusell, 2012

KIC= $\alpha$ -ketoisocaproate; P=parallel; UL=Tolerable Upper Intake Levels; X=crossover.

Table 7 summarizes chronic human clinical studies (> 1 year) reporting no adverse effects of L-leucine (as part of BCAA) in patients with various diseases. In patients with hepatic problems, supplementations of BCAA at daily doses of 28 g providing 11 g L-leucine/day for 1 year or 12-14 g BCAA providing 5.7 g L-leucine/day for 2-4 years were well tolerated with no adverse effects (Habu et al., 2009; Kawamura et al., 2009; Kobayashi et al., 2008; Kuroda et al., 2010; Marchesini et al., 2003; Nishikawa et al., 2013; Poon et al., 2004; Yoshiji et al., 2002).

In addition, a single dose of 60 g BCAA providing 24 g L-leucine was also well tolerated in healthy individuals (Gijssman et al., 2002; Scarna et al., 2002) and subjects with mania (Scarna et al., 2003). Administration of 72.5 g BCAA over a 32 hour period was also well tolerated in healthy individuals (Portier et al., 2008). GRN 308 (U.S. FDA, 2010) reported the results of the study by Verhoeven et al. (2009) in which the effects of 7.5 g/day L-leucine supplementation were tested on muscle mass and strength in 30 healthy elderly men (29 completed the study; n=15 in the L-leucine group and n=14 in the placebo group) for 3 months. No significant changes were reported in skeletal muscle mass or strength or in indexes of whole-body insulin sensitivity, blood glycated hemoglobin content, or the plasma lipid profile. However, the fasting plasma L-valine levels of 15 healthy elderly men supplemented with 2.5 g of L-leucine at each main meal (or 7.5 g/day) decreased by approximately 25% (vs. basal) within 2 weeks of treatment. The authors indicated that the clinical significance of this decline in plasma L-valine concentrations remains debatable because the plasma concentrations did not decline further after 2 weeks and remained within a normal physiologic range. Plasma L-isoleucine levels did not change significantly over the course of the study.

The body of evidence on oral administration of L-leucine in humans indicates that it can be consumed in considerable amounts without adverse effects. The daily doses that resulted in no adverse effects of L-leucine in healthy subjects (up to 12 g/day) and subjects with various diseases (5.7-11 g/day) are higher than the estimated intakes of L-leucine from the proposed food uses since EDI of all-users were 1.9 and 4.1 g at the mean and 90th percentile, respectively.

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Table 7. Chronic human clinical studies reporting no adverse effects of L-leucine in patients with various diseases

Subject	Daily dose of leucine supplement	Duration/ Design	Measured outcomes	Reference
256 patients with HCV-related $\leq 3$ cm in diameter with up to 3 nodules and a serum albumin level before RFA of $\leq 3.5$ g/dL	5.7 g L-leucine as part of 12 g BCAA; P	1-3 years, P	Overall survival and recurrence-free survival	Nishikawa et al., 2013
88 patients undergoing chemoembolization for HCC, 84 completed	6 g leucine in 20 g BCAA supplement; or no supplement	1 year, P	Morbidity, Albumin, Bilirubin, Aspartate aminotransferase, prothrombin, indocyanine green retention, nutritional status, Quality of Life, long-term survival	Poon et al, 2004
49 patients with hepatitis C-related HCC after radiofrequency ablation, 35 completed	11g leucine in 28.4 g BCAA (100 g Aminoleban EN); or normal diet	1 year, P	Event-free survival rate, liver function tests, and short form 8 scores (Quality Of Life)	Kuroda et al., 2010
174 patients with advanced cirrhosis (mean age 60 years)	7.2 g leucine in BCAA 14.4 g; placebo containing 12.6 g lactoalbumin; or maltodextrin controls	1year, P	Combined end point (death and deterioration), hospital admission, nutritional parameters, laboratory data and Child-Pugh score, anorexia and health related quality of life, advent events	Marchesini et al., 2003
65 patients (65.1 $\pm$ 6.9 years) with hepatic encephalopathy-related cirrhosis	5.7 g leucine in 14.2 g BCAA; or placebo	2 years, P	Serum albumin levels, BCAA:tyrosine ratio	Habu et al., 2009

Table 7. Chronic human clinical studies reporting no adverse effects of L-leucine in patients with various diseases, continued

Subject	Daily dose of leucine supplement	Duration/ Design	Measured outcomes	Reference
39 males 50-70years with compensated cirrhosis due to hepatitis C	5.7 g leucine in 12 g BCAA divided into 3 doses daily; or no treatment	3 years, P	Serum albumin, Fischer's ratio, onset of ascites, edema, HE, development of HCC, and adverse events	Kobayashi et al., 2008
56 patients with Child class A cirrhosis (50 completed, 62.70±10.1 years)	5.7 g leucine in 12 g BCAA divided into 3 doses daily; or no treatment	Mean 3.2 years, P	Incidence of cirrhosis-related complications, and outcome markers including Model for End-Stage Liver Disease score, Child-Turcote-Pugh score, asialoscintigraphic clearance index, serum total bilirubin, and serum albumin complications	Kawamura et al., 2009
110 patients with cirrhosis associated with HCC, 89 completed	5.7 g leucine in 12 g BCAA supplement (; a single dose of 4 mg perindopril (ACE-I); combination of perindopril/BCAA; or no treatment	4 years, P	Serum fibrosis markers, serum conc. of albumin, TGF- $\beta$ , hyaluronic acid, and insulin resistance	Yoshiji et al., 2012

HCC=hepatocellular carcinoma; HCV=hepatitis C virus; HE=hepatic encephalopathy; P=prospective; RFA=radiofrequency thermal ablation. TGF- $\beta$ = transforming growth factor- $\beta$ .

#### IV.E Other Considerations

##### IV.E.I. Safety of the Fermentation Microorganism

The fermentation microorganism is a non-pathogenic, non-toxicogenic species and strain (*Corynebacterium glutamicum*) commonly used in food processing. The FDA indicated that it had no questions in response to a GRAS Notice for lysine monhydrochloride (GRN 000414), produced using fermentation of sugars and molasses with *Corynebacterium glutamicum* (U.S. FDA, 2012).

#### **IV.E.2 Potential Antagonism of Other Amino Acids**

As discussed in GRN 308 and the IOM report (U.S. FDA, 2010; IOM, 2005), L-leucine supplementation (1 to 5% L-leucine in the diet but not 0.5%) has been shown to have antagonistic effects on other amino acids in the presence of a low protein diet (<9% casein) and resulted in growth depression (Harper et al., 1954, 1955; Benton et al., 1956; Rogers et al., 1967). The growth suppressing effects of L-leucine could be largely or fully prevented by the addition of amino acids (isoleucine, valine, phenylalanine, tryptophan, and threonine) or protein to the diet (Block, 1984). For example, Harper et al. (1984) demonstrated that high dietary levels of L-leucine suppressed growth of rats fed a low protein diet, and that the growth suppression could be prevented by supplementation of L-isoleucine and L-valine (IOM, 2005).

Other animal studies demonstrated a lack of adverse effects on growth when supplementary L-leucine was given at levels of up to approximately 6,000-8,000 mg/kg body weight/day or 10% L-leucine in diet, in conjunction with adequate dietary protein for periods ranging from 10 days to 13 weeks (Lynch et al., 2002; Tsubuku et al., 2004; Donato et al., 2006; Sugawara et al., 2007; Zhang et al., 2007).

Analysis of NHANES dataset indicated Americans' protein intake status was adequate and users of added L-leucine were likely to consume adequate levels of protein in the diet. Thus, L-leucine supplementation is not expected to cause a growth suppression problem or any other adverse effects in the American population.

##### Animal studies

In the study of Matsuzaki et al. (2005), body weight gain was significantly lower in rats fed diets containing 15 or 30% (w/w) L-leucine (equivalent to approximately 11 and 14 g/kg body weight/day, respectively) for 2 weeks compared to control animals; however, such effects did not occur at levels of 1.5, 5, or 10% L-leucine (equivalent to 1,071, 3,690, or 7,183 mg/kg body weight/day, respectively) in the diet compared the control diet (Matsuzaki et al., 2005).

Similarly, Sakai et al. (2004) reported growth suppression in Fischer rats fed diets supplemented with 15% L-leucine (approximately 12,400 mg/kg body weight; duration not reported). However, rats were fed diets supplemented with 10% L-leucine (approximately 8,200 mg/kg body weight/day) for 1 week had no statistically significant changes in body weight.

##### Protein intake status in Americans

Table 8 presents daily protein intakes at mean, 10<sup>th</sup> percentile, and 90<sup>th</sup> percentile estimated by the IOM. The 10<sup>th</sup> percentile intake levels exceeded the EARs in all age/gender. The data indicate that Americans' protein intake status is adequate and users of added L-leucine are likely to consume adequate levels of protein in the diet. Thus, L-leucine supplementation is not expected to cause a growth suppression problem or any other adverse effects in the American population.

Table 8. Americans' Daily Protein Intakes at Mean, 10<sup>th</sup> Percentile, and 90<sup>th</sup> Percentile and EAR for Protein

	Daily intake of protein, g/day; Percentile				EAR for protein	
	5th	10th	50th	90th	g/day, IOM <sup>1</sup>	g/day, Elango et al., 2010 <sup>2</sup>
Infants, 7-12 mo	14.4	16.5	26.5	42.5	9.9	--
Children, 1-3	31.3	34.9	49.8	68.4	13	--
Children 4-8 y	40.8	44.7	61.2	81.9	19	--
Boys, 9-13y	54.0	59.0	78.0	101.0	34	--
Girls, 9-13 y	44.4	48.5	64.0	83.6	34	--
14-18 y, boys	62.0	69.0	97.0	132.0	45	--
14-18 y, girls	44.4	48.8	65.4	85.3	38	--
Adult men, 19-30 y	62	70	101	142	46.2	--
Adult women, 19-30 y	40	44	62	84	38.0	--
Adult men, 31-50y	61	67	97	135	46.2	--
Adult women, 31-50y	41	46	64	86	38.0	--
Adult men, 51-70y	53	59	85	117	46.2	--
Adult women, 51-70y	40	44.3	60.8	80.4	38.0	--
Adult men, 71y+	42	48	71	99	46.2	65.1
Adult women, 71y+	34	38.2	55.2	76	38.0	--

Source: <sup>1</sup>IOM, 2005; <sup>2</sup>Elango et al., 2010.

The recent Dietary Reference Intake (DRI) recommendations for mean intakes of 0.66 g/kg body weight/day of high-quality protein in adult humans are based on a meta-analysis of nitrogen balance studies using single linear regression analysis (IOM, 2005). Elango et al. (2010) reanalyzed existing nitrogen balance studies using two-phase linear regression analysis and obtained mean protein requirements of 0.91 g/kg body weight/day. The two-phase linear regression analysis is considered more appropriate for biological analysis of dose-response curves. Considering the inherent problems associated with the nitrogen balance method, Elango et al. (2012a, 2012b) developed an alternative method, the indicator amino acid oxidation technique, to determine protein requirements. The mean requirements in adult men were determined to be 0.93 g/kg body weight/day (41% higher than the IOM value) corresponding to 65.1 g protein/day. Even if the highest protein requirement value is used, there is no evidence that protein intake status in Americans is inadequate. Thus, supplementation of L-leucine is not expected to cause an amino acid imbalance problem or any other adverse effects in the American population.

## V. SAFETY DETERMINATION

Numerous human and animal studies have reported the health benefits of L-leucine, an essential amino acid, with no major adverse effects. INNOBIO® uses a Hazard Analysis and Critical Control Point (HACCP)-controlled manufacturing process and rigorously tests its final production batches to verify adherence to quality control specifications. There is broad-based and widely disseminated knowledge concerning the chemistry of L-leucine. This GRAS determination is based on the data and information generally available and consented opinion about the safety of L-leucine. The literature indicates that L-leucine offers consumers health benefits without serious adverse effects.

The following safety evaluation fully considers the composition, intake, nutritional, microbiological, and toxicological properties of L-leucine as well as appropriate corroborative data.

1. The intended uses of L-leucine are the same as those described in GRN 308 and will substitute for currently marketed L-leucine products. L-Leucine is proposed for use as an ingredient in milk and non-milk based meal replacements, sports and isotonic beverages, vitamin enhanced waters, and meal replacement bars, at levels of 0.5-3.0 g/serving. Consequently, INNOBIO® notes that its uses will not result in any exposure beyond what was previously estimated. The proposed food uses result in an EDI for users (mean of 1.9 g/person/day or 28 mg/kg body weight/day and 90th percentile intake of 4.1 g/person/day or 64 mg/kg body weight/day) at levels significantly below those associated with any side effects.
2. In a 13-week rat toxicity study, the highest level studied for L-leucine product, 5% mixed into the diet, was established as the NOAEL. This NOAEL corresponded to a daily intake of 3,333 or 3,835 mg L-leucine/kg body weight/day for female and male rats, respectively, in addition to the amount provided by the basal diet. In a reproductive and developmental study, the highest dose administered by oral gavage, 1,000 mg/kg body weight/day, was established as the NOAEL for female reproductive function and embryo-fetal development. In addition, growth studies also showed that rats were fed diets supplemented with 10% L-leucine (approximately 8,200 mg/kg body weight/day) for 1-2 weeks had no statistically significant changes in body weight.
3. Human clinical studies reported no adverse effects associated with single oral supplementation of up to 60 g/day of BCAAs (providing 24 g L-leucine) in healthy subjects and bipolar subjects. Repeated dose studies showed that daily doses up to 12 g were well tolerated with no adverse effects in health subjects and subjects with type 2 diabetes. In patients with hepatic problems, long term supplementation of L-leucine at daily doses of 6-11g L-leucine (in 14-28 g BCAA) for 1 year and 5.7 g L-leucine (in 12 g BCAA/day) for 2-3 years were well tolerated with no adverse effects.
4. The IOM has not established the UL of protein or individual amino acids since for some well studied amino acids, there were no adverse effects reported at the highest dose tested in long term studies. The IOM also noted that many animal studies of amino acid toxicity

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were conducted with diets deficient in protein and that less emphasis was placed on these studies than those with adequate protein diets because of creation of amino acid imbalances. Recently, the ICAAS has proposed an upper limit of safe intake for L-leucine for healthy adults as 0.53 g/kg/day, which corresponds to 37.1 g/day in a 70 kg adult. The NHANES analysis shows that Americans' protein intake status is considered adequate. Thus, BCAA antagonism would not be expected from the amount of L-leucine estimated to be consumed from the proposed food uses.

5. Authoritative government agencies including the U.S. FDA (2010) (GRN 308) have not raised questions regarding the safety of L-leucine as currently used in food. Since the FDA's last review of L-leucine in 2009-2010, no literature contradicting the agency's prior reviews has been published.
6. The compositional data and product properties are consistent with carefully controlled cGMP production and purification. L-leucine contains no impurities or contaminants of concern for human health. L-leucine is produced in accordance with cGMP and meets appropriate food-grade specifications. L-Leucine is obtained via fermentation using *Corynebacterium glutamicum* which has been safely used in industrial production of amino acids for a long time. INNOBIO® has established chemical and microbiological specifications consistent with other food-grade materials. Lot samples are routinely evaluated to verify compliance with the specifications.
7. Several reviews by experts in the field also have documented the safety of L-leucine.

## **VI. CONCLUSIONS AND GENERAL RECOGNITION OF THE SAFETY OF L-LEUCINE**

The intended use of L-leucine has been determined to be safe through scientific procedures as set forth in 21 CFR 170.3(b), thus satisfying the so-called “technical” element of the GRAS determination. In addition, because this safety evaluation was based on generally available and widely accepted data and information, it also satisfies the so-called “common knowledge” element of a GRAS determination.

On behalf of INNOBIO® Limited (INNOBIO®), expert panel members, Susan Cho, Ph.D., Paul Pencharz, M.D., Ph.D., and Steven Heymsfield, M.D., have independently evaluated the materials summarized in the L-leucine Generally Recognized as Safe (GRAS) report. These individuals are qualified by scientific training and experience to evaluate the safety of substances intended to be added to foods. They have critically reviewed and evaluated the publicly available information summarized in this document and have individually and collectively concluded that L-leucine, produced consistent with cGMP and meeting the specifications described herein, is safe under its intended conditions of use. The Panel further unanimously concludes that these uses of L-leucine are GRAS based on scientific procedures, and other experts qualified to assess the safety of food and food ingredients would concur with these conclusions.

It is also INNOBIO’s opinion that the proposed use of L-leucine is not only safe within the terms of the Federal Food, Drug, and Cosmetic Act (meeting the standard of reasonable certainty of no harm), but it is also *Generally Recognized as Safe* (GRAS) according to Title 21 Code of Federal Regulations (21 CFR) according to consensus among experts.

## REFERENCES

- Benton DA, Harper AE, Spivey HE, Elvehjem CA. Leucine, L-isoleucine and L-valine relationships in the rat. *Arch Biochem Biophys.* 1956; 60:147-155.
- Björkman MP, Pilvi TK, Kekkonen RA, Korpela R, Tilvis RS. Similar effects of L-leucine rich and regular dairy products on muscle mass and functions of older polymyalgia rheumatica patients: a randomized crossover trial. *J Nutr Health Aging.* 2011;15:462-467.
- Block KP, Harper AE. L-valine metabolism in vivo. effects of high dietary levels of L-leucine and isoleucine. *Metabolism.* 1984;33:559-566.
- Campos-Ferraz PL, Bozza T, Nicastró H, Lancha AH Jr. Distinct effects of leucine or a mixture of the branched-chain amino acids (leucine, isoleucine, and valine) supplementation on resistance to fatigue, and muscle and liver-glycogen degradation, in trained rats. *Nutrition.* 2013;29:1388-1394.
- Casperson SL, Sheffield-Moore M, Hewlings SJ, Paddon-Jones D. L-leucine supplementation chronically improves muscle protein synthesis in older adults consuming the RDA for protein. *Clin Nutr.* 2012;31:512-519.
- CDC. 2006. *Analytical and Reporting Guidelines. The National Health and Nutrition Examination Survey (NHANES).* Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: [http://www.cdc.gov/nchs/data/nhanes/nhan0e3s\\_04/nhanes\\_analytic\\_guidelines\\_dec2005.pdf](http://www.cdc.gov/nchs/data/nhanes/nhan0e3s_04/nhanes_analytic_guidelines_dec2005.pdf).
- Chen H, Simar D, Ting JH, Erkelens JR, Morris MJ. L-leucine improves glucose and lipid status in offspring from obese dams, dependent on diet type, but not caloric intake. *J Neuroendocrinol.* 2012;24:1356-1364.
- Cota D, Proulx K, Smith KA, Kozma SC, Thomas G, Woods SC, Seeley RJ. Hypothalamic mTOR signaling regulates food intake. *Science.* 2006;312:927-930.
- Crowe MJ, Weatherson JN, Bowden BF. Effects of dietary L-leucine supplementation on exercise performance. *Eur J Appl Physiol.* 2006;97:664-672.
- Cynober L, Bier DM, Kadowaki M, Morris SM Jr, Renwick AG. A proposal for an upper limit of L-leucine safe intake in healthy adults. *J Nutr.* 2012;142:2249S-2250S.
- Donato J Jr, Pedrosa RG, Cruzat VF, Pires IS, Tirapegui J. Effects of L-leucine supplementation on the body composition and protein status of rats submitted to food restriction. *Nutrition.* 2006;22:520-527.
- Elango R, Chapman K, Rafii M, Ball RO, Pencharz PB. Determination of the tolerable upper intake level of leucine in acute dietary studies in young men. *Am J Clin Nutr.* 2012a;96:759-767.

## L-leucine GRAS

Elango R, Ball RO, Pencharz PB. Recent advances in determining protein and amino acid requirements in humans. *Br J Nutr.* 2012b;108 Suppl 2:S22-30.

Elango R, Humayun MA, Ball RO, Pencharz PB. Evidence that protein requirements have been significantly underestimated. *Curr Opin Clin Nutr Metab Care.* 2010;13:52-57.

Etzel MR. Manufacture and use of dairy protein fractions. *J Nutr.* 2004;134:996S-1002S.

Gijssman HJ, Scarnà A, Harmer CJ, McTavish SB, Odontiadis J, Cowen PJ, Goodwin GM. A dose-finding study on the effects of branch chain amino acids on surrogate markers of brain dopamine function. *Psychopharmacology (Berl).* 2002;160:192-197.

Habu D, Nishiguchi S, Nakatani S, Lee C, Enomoto M, Tamori A, Takeda T, Ohfuji S, Fukushima W, Tanaka T, Kawamura E, Shiomi S. Comparison of the effect of BCAA granules on between decompensated and compensated cirrhosis. *Hepatogastroenterology.* 2009;56:1719-1723.

Harper AE, Benton DA, Winje ME, Elvehjem CA. Leucine-isoleucine antagonism in the rat. *Arch Biochem Biophys.* 1954;51:523-524.

Harper AE, Benton DA, Elvehjem CA. L-Leucine, an L-isoleucine antagonist in the rat. *Arch Biochem.* 1955;57:1-12.

Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. *Annu Rev Nutr.* 1984;4:409-454.

Imamura W, Yoshimura R, Takai M, Yamamura J, Kanamoto R, Kato H. Adverse effects of excessive L-leucine intake depend on dietary protein intake: a transcriptomic analysis to identify useful biomarkers. *J Nutr Sci Vitaminol (Tokyo).* 2013;59:45-55.

IOM. 2005. Protein and Amino Acids. In: *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients)*. Washington, DC: Institute of Medicine (IOM), National Academy Press (NAP), pp. 589-768. Available at: <http://www.nap.edu/openbook.php?recordid=104908page=589>.

Ispoglou T, King RF, Polman RC, Zanker C. Daily L-leucine supplementation in novice trainees during a 12-week weight training program. *Int J Sports Physiol Perform.* 2011;6:38-50.

Jackman SR, Witard OC, Jeukendrup AE, Tipton KD. Branched-chain amino acid ingestion can ameliorate soreness from eccentric exercise. *Med Sci Sports Exerc.* 2010;42:962-970.

Kakizoe T, Nishio Y, Honma Y, Nijima T, Sugimura T. 1983. L-Isoleucine and L-leucine are promoters of bladder cancer in rats. In: Fujiki H, Hecker E, Moore RE, Sigimura T, Weinstein IB, editors. *Cellular interactions by Environmental Tumor Promoters: Proceedings of the 14th International Symposium of the Princess Takamatsu Cancer Research Fund. (Princess Takamatsu Symposia)*. Tokyo, Japan: Japan Scientific Societies Press, pp. 373-380.

## L-leucine GRAS

Kawamura E, Habu D, Morikawa H, Enomoto M, Kawabe J, Tamori A, Sakaguchi H, Saeki S, Kawada N, Shiomi S. A randomized pilot trial of oral branched-chain amino acids in early cirrhosis: validation using prognostic markers for pre-liver transplant status. *Liver Transpl.* 2009;15:790-797.

Kirby TJ, Triplett NT, Haines TL, Skinner JW, Fairbrother KR, McBride JM. Effect of L-leucine supplementation on indices of muscle damage following drop jumps and resistance exercise. *Amino Acids.* 2012;42:1987-96.

Kobayashi M, Ikeda K, Arase Y, Suzuki Y, Suzuki F, Akuta N, Hosaka T, Murashima N, Saitoh S, Someya T, Tsubota A, Kumada H. Inhibitory effect of branched-chain amino acid granules on progression of compensated liver cirrhosis due to hepatitis C virus. *J Gastroenterol.* 2008;43:63-70.

Kraus AL, Munro IC, Orr JC, Binder RL, LeBoeuf RA, Williams GM. Benzoyl peroxide: an integrated human safety assessment for carcinogenicity. *Regul Toxicol Pharmacol.* 1995;21:87-107.

Kuroda H, Ushio A, Miyamoto Y, Sawara K, Oikawa K, Kasai K, Endo R, Takikawa Y, Kato A, Suzuki K. Effects of branched-chain amino acid-enriched nutrient for patients with hepatocellular carcinoma following radiofrequency ablation: a one-year prospective trial. *J Gastroenterol Hepatol.* 2010;25:1550-1555.

Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Hartgens F, Wodzig WK, Saris WH, van Loon LJ. Prolonged L-leucine supplementation does not augment muscle mass or affect glycemic control in elderly type 2 diabetic men. *J Nutr.* 2011;141:1070-1076.

Li X, Wang X, Liu R, Ma Y, Guo H, Hao L, Yao P, Liu L, Sun X, He K, Cao W, Yang X. Chronic leucine supplementation increases body weight and insulin sensitivity in rats on high-fat diet likely by promoting insulin signaling in insulin-target tissues. *Mol Nutr Food Res.* 2013;57:1067-1079.

Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC. Tissue-specific effects of chronic dietary L-leucine and norleucine supplementation on protein synthesis in rats. *Am J Physiol Endocrinol Metab.* 2002;283:E824-E835.

Marchesini G, Bianchi G, Merli M, Amodio P, Panella C, Loguercio C, Rossi Fanelli F, Abbiati R; Italian BCAA Study Group. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. *Gastroenterology.* 2003;124:1792-1801.

Matsuzaki K, Kato H, Sakai R, Toue S, Amao M, Kimura T. Transcriptomics and metabolomics of dietary L-leucine excess. *J Nutr.* 2005;135: Suppl:1571 S-1575S.

Mawatari K, Katsumata T, Uematsu M, Katsumata T, Yoshida J, Smriga M, Kimura T. Prolonged oral treatment with an essential amino acid L-leucine does not affect female

## L-leucine GRAS

reproductive function and embryo-fetal development in rats. *Food Chem Toxicol.* 2004;42:1505-11.

Mero A, Pitkänen H, Oja SS, Komi PV, Pöntinen P, Takala T. L-leucine supplementation and serum amino acids, testosterone, cortisol and growth hormone in male power athletes during training. *J Sports Med Phys Fitness.* 1997;37:137-145.

Mero A, Leikas A, Knuutinen J, Hulmi JJ, Kovanen V. Effect of strength training session on plasma amino acid concentration following oral ingestion of leucine, BCAAs or glutamine in men. *Eur J Appl Physiol.* 2009;105:215-223.

Millward DJ. Knowledge gained from studies of L-leucine consumption in animals and humans. *J Nutr.* 2012 Dec;142:2212S-2219S.

Nishikawa H, Osaki Y, Iguchi E, Koshikawa Y, Ako S, Inuzuka T, Takeda H, Nakajima J, Matsuda F, Sakamoto A, Henmi S, Hatamaru K, Ishikawa T, Saito S, Nasu A, Kita R, Kimura T. The effect of long-term supplementation with branched-chain amino acid granules in patients with hepatitis C virus-related hepatocellular carcinoma after radiofrequency thermal ablation. *J Clin Gastroenterol.* 2013;47:359-366.

Nishio Y, Kakizoe T, Ohtani M, Sato S, Sugimura T, Fukushima S. L-isoleucine and L-leucine: Tumor promoters of bladder cancer in rats. *Science.* 1986;231:843-845.

PDRNS. 2008. Branch-chain amino acids (L-leucine, L-isoleucine, L-valine). In: *PDRNS for Nutritional Supplements*, 2nd edition. Montvale (NJ): Physicians' Desk Reference (PDR), pp. 100-104.

Pencharz PB, Russell RM. Application of Key Events Dose Response Framework to defining the upper intake level of leucine in young men. *J Nutr.* 2012;142:2225S-2226S.

Pencharz PB, Elango R, Ball RO. Determination of the tolerable upper intake level of leucine in adult men. *J Nutr.* 2012;142:2220S-2224S.

Pereira MG, Baptista IL, Carlassara EO, Moriscot AS, Aoki MS, Miyabara EH. Leucine supplementation improves skeletal muscle regeneration after cryolesion in rats. *PLoS One.* 2014 ;9:e85283.

Pitkänen HT, Oja SS, Rusko H, Nummela A, Komi PV, Saransaari P, Takala T, Mero AA. L-leucine supplementation does not enhance acute strength or running performance but affects serum amino acid concentration. *Amino Acids.* 2003;25:85-94.

Poon RT, Yu WC, Fan ST, Wong J. Long-term oral branched chain amino acids in patients undergoing chemoembolization for hepatocellular carcinoma: a randomized trial. *Aliment Pharmacol Ther.* 2004;19:779-788.

## L-leucine GRAS

Rogers QR, Tannous RI, Harper AE. Effects of excess L-leucine on growth and food selection. *J Nutr.* 1967; 91:561-572

Rosenthal J, Angel A, Farkas J. Metabolic fate of leucine: a significant sterol precursor in adipose tissue and muscle. *Am J Physiol.* 1974;226:411-418.

Sakai R, Miura M, Amao M, Kodama R, Toue S, Noguchi Y. Potential approaches to the assessment of amino acid adequacy in rats: a progress report [includes discussion]. *J Nutr.* 2004;134:1651S-1655S, discussion 1664S-1666S, 1667s-1672s.

Sargentini NJ, Smith KC. Mutagenesis by normal metabolites in *Escherichia coli* phenylalanine mutagenesis is dependent on error-prone DNA repair. *Mutat Res.* 1986;161:113-118.

Scarna A, Gijsman HJ, McTavish SF, Harmer CJ, Cowen PJ, Goodwin GM. Effects of a branched-chain amino acid drink in mania. *Br J Psychiatry.* 2003;182:210-213.

Scarnà A, Gijsman HJ, Harmer CJ, Goodwin GM, Cowen PJ. Effect of branch chain amino acids supplemented with tryptophan on tyrosine availability and plasma prolactin. *Psychopharmacology (Berl).* 2002;159:222-223.

Shimomura Y, Yamamoto Y, Bajotto G, Sato J, Murakami T, Shimomura N, Kobayashi H, Mawatari K. Nutraceutical effects of branched-chain amino acids on skeletal muscle. *J Nutr.* 2006;136:529S-532S.

Stock MS, Young JC, Golding LA, Kruskall LJ, Tandy RD, Conway-Klaassen JM, Beck TW. The effects of adding leucine to pre and postexercise carbohydrate beverages on acute muscle recovery from resistance training. *J Strength Cond Res.* 2010;24:2211-2219.

Sugawara T, Ito Y, Nishizawa N, Nagasawa T. Supplementation with dietary L-leucine to a protein-deficient diet suppresses myofibrillar protein degradation in rats. *J Nutr Sci Vitaminol.* 2007;53:552-555.

Swendseid ME, Villalobos J, Figueroa WS, Drenick EJ. The effects of test doses of leucine, L-isoleucine or L-valine on plasma amino acid levels. The unique effect of L-leucine *Am J Clin Nutr.* 1965;17:317-321.

Torres-Leal FL, Fonseca-Alaniz MH, Teodoro GF, de Capitani MD, Vianna D, Pantaleão LC, Matos-Neto EM, Rogero MM, Donato J Jr, Tirapegui J. L-leucine supplementation improves adiponectin and total cholesterol concentrations despite the lack of changes in adiposity or glucose homeostasis in rats previously exposed to a high-fat diet. *Nutr Metab (Lond).* 2011;8:62-71.

Tsubuku S, Hatayama K, Katsumata T, Nishimura N, Mawatari K, Smriga M et al. Thirteen-week oral toxicity study of branched-chain amino acids in rats. *Int J Toxicol.* 2004;23:119-126.

## L-leucine GRAS

U.S. FDA. 2010. Agency Response Letter GRAS Notice No. GRN 000308 (L-leucine). College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN).

U.S. FDA. 2009. U.S. Code of Federal Regulations (CFR). Title 21-Food and Drugs (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: <http://www.access.gpo.gov/cqi-bin/cfr/asmbl/bq/cfr?title=200821> [See Table for CFR sections used].

U.S. FDA. 2012. Agency Response Letter GRAS Notice No. GRN 000414 (L-lysine monohydrochloride). College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN). Available at: <http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm332206.htm>

U.S. FDA. 1993. Appendix I. Table 14. Conversion table for test chemical treatment doses used in PAFA. In: Priority Based Assessment of Food Additives (PAFA) Database. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), p. 58.

U.S. FDA. Substances generally recognized as safe; Proposed rule (21 CFR Parts 170, 184, 186, and 570) [Docket No. 97N-01031. Fed Regist (US). 1997; 62(74):18937-18964.

USDA. 2006. USDA National Nutrient Database for Standard Reference. Release 19. Available at: <http://www.ars.usda.gov/Services/docs.htm?docid=8964>.

Verhoeven S, Vanschoonbeek K, Verdijk LB, Koopman R, Wodzig WK, Dendale P et al. Long-term L-leucine supplementation does not increase muscle mass or strength in healthy elderly men. *Am J Clin Nutr*. 2009; 89:1468-1475.

Walker TB, Smith J, Herrera M, Lebeque B, Pinchak A, Fischer J. The influence of 8 weeks of whey-protein and L-leucine supplementation on physical and cognitive performance. *Int J Sport Nutr Exerc Metab*. 2010;20:409-417.

Witham WG, Yester KA, McGaffin KR. A high L-leucine diet mitigates cardiac injury and improves survival after acute myocardial infarction. *Metabolism*. 2013;62:290-302.

Xie XL, Wei M, Yunoki T, Kakehashi A, Yamano S, Kato M, Wanibuchi H. Long-term treatment with L-isoleucine or L-leucine in AIN-93G diet has promoting effects on rat bladder carcinogenesis. *Food Chem Toxicol*. 2012;50:3934-3940.

Xing W, Na R. Amino acids excess increase SCEs in human lymphocytes. *Mutat Res*. 1996;372:75-78.

Yoshiji H, Noguchi R, Ikenaka Y, Kaji K, Aihara Y, Douhara A, Yamao J, Toyohara M, Mitoro A, Sawai M, Yoshida M, Morioka C, Fujimoto M, Uemura M, Fukui H. Combination of

## L-leucine GRAS

branched-chain amino acid and angiotensin-converting enzyme inhibitor improves liver fibrosis progression in patients with cirrhosis. *Mol Med Rep.* 2012;5:539-544.

Zemel MB, Bruckbauer A. Effects of a L-leucine and pyridoxine-containing nutraceutical on fat oxidation, and oxidative and inflammatory stress in overweight and obese subjects. *Nutrients.* 2012;4:529-541.

Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH. Increasing dietary L-leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. *Diabetes.* 2007; 56:1647-1654.

## **APPENDIX A.**

### **EXPERT PANEL OPINION THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF L-LEUCINE FOR USE AS A FOOD INGREDIENT**

#### **INTRODUCTION**

The undersigned, an independent panel of experts, qualified by their scientific training and national and international experience to evaluate the safety of food ingredients (the “Expert Panel”), was specially convened by INNOBIO® Limited (INNOBIO®) and asked to evaluate the safety and Generally Recognized as Safe (GRAS) status of the proposed use of L-leucine as a food ingredient.

A comprehensive search of the literature bearing on the safety of L-leucine was performed by INNOBIO®. The results of the literature search were summarized in the safety dossier “GRAS Determination of L-Leucine for Use as a Food Ingredient” for consideration by the Expert Panel.

The Expert Panel reviewed the safety documentation, as well as other available data and information that the Panel members believed to be pertinent to the safety of L-leucine under the conditions of intended use as a food ingredient. In addition, the Expert Panel reviewed the methods of manufacture and specifications for L-leucine, analytical data confirming compliance with specifications, and the proposed use and use levels of the proposed food ingredient. The Expert Panel independently and unanimously concluded that L-leucine, produced consistent with current good manufacturing practice (cGMP) and meeting appropriate specifications, is safe for its intended use. The Expert Panel further concluded that this intended use is GRAS based on scientific procedures. It is also the opinion of the Expert Panel that other qualified experts would concur with our conclusions. Summarized below is the Panel’s scientific analysis supporting its conclusions.

#### **DESCRIPTION**

##### **Common or Trade Name**

The common name of the notified substance is L-leucine. Trade name is INNOBIO® Vegan Instantized L-Leucine Powder.

##### **Background**

L-leucine is an indispensable or essential amino acid, whose skeleton can not be synthesized from simpler molecules in humans, and it therefore must be ingested (Institute of Medicine [IOM], 2005). L-leucine, along with L-isoleucine and L-valine, are classified as branched chain amino acids (BCAA) that differ from most other indispensable amino acids in that enzymes initially responsible for their catabolism are found primarily in extrahepatic tissues. L-leucine is also classified as a hydrophobic amino acid due to its aliphatic isobutyl side chain and is a major component of the subunits in ferritin, astacin and other 'buffer' proteins.

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Supplements of L-leucine and the BCAA are advocated and widely used in relation to performance and in various clinical situations to maintain muscle mass during weight loss and in the elderly (Millward, 2012). Muscle mass is regulated by the synthesis and degradation of muscle protein, which in turn are affected by aging, several catabolic diseases, and malnutrition. Amino acids, particularly leucine, are known to stimulate muscle protein synthesis and suppress muscle protein degradation (Sugawara et al., 2007). Thus, L-leucine has earned more attention on its own as a catalyst for muscle growth and muscular insurance.

The estimated average requirement (EAR) of L-leucine is 34 mg/kg body weight/day which corresponds to 2.38 g/day for a 70 kg adult (Institute of Medicine [IOM], 2005). The IOM has not established Tolerable Upper Intake Levels (UL) for L-leucine and other indispensable amino acids. The UL is defined as the highest average daily intake of a nutrient that is likely to pose no risk of adverse health effects for nearly all persons in the general population.

In stating the reasons why the ULs have not been established for individual amino acids, the IOM states the following:

- “There is no evidence that amino acids derived from usual or even high intakes of protein from food stuffs present any risk.”
- “More emphasis was placed on observations of adverse effects in humans than on effects observed in animals, and pharmacokinetic studies were sought to bridge potential differences between animals and humans.”
- “Many animal studies of amino acid toxicity were conducted with diets deficient in protein. Less emphasis was placed on these studies than those with adequate protein diets because of creation of amino acid imbalances.”
- “For some well studied amino acids, there were no adverse effects reported at the highest dose tested in long term studies. In such cases it was not possible to establish a Lowest Observed-Adverse-Effect Level (LOAEL) or a No Observed-Adverse-Effect Level (NOAEL) that was supported by toxicity data. Under these circumstances it was not possible to establish a UL in keeping the criteria and procedures required by the UL model.”

Recently, the International Council for Amino Acid Sciences (ICAAS) (Cynober et al., 2012; Elango et al., 2012; Pencharz et al., 2012; Pencharz and Russell, 2012) proposed an upper limit of safe intake for L-leucine for healthy adults as 0.53 g/kg body weight/day, which corresponds to 37.1 g/day in a 70 kg adult.

## CHEMISTRY AND COMPOSITION

**Chemical Name:** (2S)-2-amino-4-methylpentanoic acid; L-2-amino-4-methylvaleric

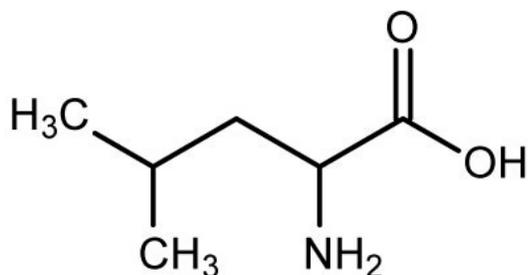
**Chemical Abstracts Service (CAS) Number:** 61 -90-5

**Empirical Formula and Formula Weight:** C<sub>6</sub>H<sub>13</sub>NO<sub>2</sub>

**Molecular Weight:** 131.17 g/mol

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Figure 1 shows chemical structure of L-leucine.



### MANUFACTURING PROCESS

For L-leucine production, sterilized glucose and corn steep liquor are fermented with *Corynebacterium glutamicum*, which has been safely used in industrial production of amino acids for a long time, at 28-30 °C for 2-3 days. After fermentation, the broth is passed through an ion exchange resin. The filtrate is concentrated and decolorized. Food grade lecithin and water are added to decolorized L-leucin concentrate. This mixture is emulsified in a high-speed shear mixer and is dried at 80~120°C to obtain a dried powder. The dried powder is passed through a sieve (40 mesh) to obtain vegan instantized L-leucine powder.

All equipment and materials used in the production process have a safe history of use in food processing. Process tanks and lines are cleaned with sodium hydroxide and hydrogen peroxide following standard procedures common to the dairy industry. All ion exchange resins used for chromatographic purification comply with 21 C.F.R. § 173.25. Celite is cleared under 27 CFR § 24.243 (Filtering aids). Similar uses of activated carbon are considered GRAS for purification and clarification of wine as per 27 CFR §24.246. All processing aids used in the manufacturing process are considered safe and suitable.

Current Good Manufacturing Practice (cGMP), which includes Hazard Analysis and Critical Control Point (HACCP) risk analysis as well as hygiene and transport standards, is applied throughout the manufacturing process; continuous monitoring is used for control. Sampling schemes and analysis schedules are applied to raw materials, products in all stages of processing, and end products. The finished product is routinely tested to ensure that it meets specifications. Parameters are set to ensure a high degree of purity that is consistent with its use as a food ingredient.

### Stability of L-Leucine Preparations

INNOBIO<sup>®</sup> Vegan Instantized L-Leucine Powder is stable at ambient temperature for up to 2 years.

### Current Regulatory Status and Background Dietary Intake of L-Leucine

L-Leucine, free, hydrated, or anhydrous or as the hydrochloride, sodium or potassium salts, is permitted for use as a special dietary and nutritional additive to significantly improve the biological quality of the total protein in a food containing naturally occurring primarily-intact

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protein that is considered a significant dietary protein source (Title 21 Code of Federal Regulations [21 CFR] 172.320) (U.S. Food and Drug Administration [FDA], 2009). As a food additive, L-leucine should meet the specifications established by the Food Chemicals Codex (FCC), National Academy of Sciences/National Research Council (NAS/NRC), and the amount of L-leucine added for nutritive purposes plus the amount naturally present in free and combined (as protein) form should not exceed 8.8% by weight of the total protein of the finished food. L-Leucine also may be used as a lubricant in the manufacture of aspartame or neotame tablets for sweetening hot beverages at levels not to exceed 3.5% of the tablet weight (21 CFR 172.804; 21 CFR 172.829; U.S. FDA, 2009). Additionally, L-leucine is deemed to be GRAS by the Flavor and Extract Manufacturers' Association (FEMA) under specific conditions of use.

### **INTENDED USES AND EXPOSURE ESTIMATES**

#### **Food sources of L-Leucine**

Foods that are rich in L-leucine include soy, lentils, beef, fish, chicken, peanuts, almonds, eggs, and milk (USDA, 2006).

#### **Intended Use**

INNOBIO<sup>®</sup> intends to market L-Leucine as a food ingredient in selected food categories, including milk and non-milk based meal replacements, sports and isotonic beverages, vitamin enhanced waters, and meal replacement bars at levels that provide 0.5 to 3 g L-leucine/serving.

#### **Self Limiting Levels of Use**

The use of L-leucine is limited by its slightly bitter taste. Thus, the use of L-leucine in foods at upper use-levels is largely self-limiting based on its organoleptic properties.

#### **Exposure Estimates**

##### Estimated Daily Intakes (EDI) from before the intended use

Dairy products are a rich source of L-leucine which occurs in the diet as a component of protein (USDA, 2006). The IOM (2005) estimated that the mean and 90th percentile total population background dietary intakes of L-leucine in Americans are 6.08 and 8.90 g/person/day, respectively and that the highest 99<sup>th</sup> percentile intakes of L-leucine was in men ages 51 to 70 years at 14.1 g/person/day. British adults had similar intake patterns. In British adults, median and 90th percentile intake values for L-leucine were 108 and 138 mg/kg body weight/day (6.5 and 8.3 g/d for 60 kg body weight) or 8.3% of the protein intake (Millward et al., 2012).

##### EDI under the intended use

The intended use of L-leucine is the same as that described in GRN 308 and will substitute for currently marketed L-leucine products. L-Leucine component in BCAA is proposed for use as an ingredient in milk and non-milk based meal replacements, sports and isotonic beverages, vitamin enhanced waters, and meal replacement bars, at levels of 0.5-3.0 g/serving. Consequently, INNOBIO<sup>®</sup> notes that its uses will not result in any exposure beyond what was previously estimated. Based on previous analysis of National Health and Nutrition Examination Survey (NHANES) 2005-2006 (CDC, 2006; GRN 308, U.S.FDA, 2010), the proposed food uses would result in an estimated daily intake for users (mean of 1.9 g/person/day or 28 mg/kg body

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weight/day and 90th percentile intake of 4.1 g/person/day or 64 mg/kg body weight/day) at levels significantly below those associated with any side effects. On an individual population basis, the greatest all-user intake of added L-leucine was found in female adults at 2.5 g/person/day. On a body weight basis, the highest all-user intake of added L-leucine occurred in infants (49 mg/kg body weight/day).

## SAFETY DATA

### **Mutagenicity and genotoxicity studies**

In a mutagenesis assay conducted by Sargentini and Smith (1986), L-leucine at a concentration of 2 mM (234 pg/mL) was not mutagenic in *E. coli* strains *uvr6*, *uvrB umu C*, and *uvrB LexA* in the absence of metabolic activation. In another study, L-leucine at concentrations of 10, 50, or 100 mg/mL produced slight, yet significant increases in sister chromatid exchanges (SCEs) in human lymphocytes compared to a control group (Xing and Na, 1996). The authors suggested the elevated frequencies of SCE were considered to be metabolic rather than genotoxic responses, since the effect was not dose-dependent.

### **Animal toxicity studies**

Tsubuku et al. (2004) evaluated the toxicological and behavioral effects of L-isoleucine, L-valine, and L-leucine in male and female Sprague-Dawley rats. The amino acids were incorporated into a standard diet at doses equal to 1.25%, 2.5%, and 5.0% (w/w). A control group of rats received a standard diet. All diets were administered ad libitum for 13 consecutive weeks. To examine stability of any potential effects, the administration period was followed by a 5-week recovery period, during which only the standard diet was provided to all animals. No treatment related abnormalities were found in rats fed a leucine-supplemented diet. The NOAEL for the L-leucine is 5.0% in rodents for both genders (Tsubuku et al., 2004). This was the highest level fed, which is equivalent to 3,333 mg/kg body weight/day for males and 3,835 mg/kg body weight/day for females.

One developmental and reproductive toxicity study of L-leucine (Chen et al., 2012) was published since the last review of 2009-2010. The study of Chen et al. (2012) found that the NOAEL for was 1.5% in drinking water, the highest level tested. For reproductive and development al toxicity, the previously found NOAEL of 1,000 mg/kg body weight/day (Mawatari et al., 2004) is still valid.

Results from additional rodent studies on L-leucine, which were not conducted primarily to assess safety but included measurements of some safety-related endpoints, corroborate the safety information provided from animal toxicity studies. In these studies, L-leucine was consumed in the diet or drinking water at levels of up to 5% in diet (Donato et al., 2006; Lynch et al., 2002; Matsuzaki et al., 2005; Sugawara et al., 2007; Witham et al., 2013; Zhang et al., 2007).

### **Human clinical studies**

The IOM (2005) has not established UL for L-leucine. Recently, the ICAAS (Cynober et al., 2012) proposed an upper limit of safe intake for L-leucine for healthy adults as 0.53 g/kg body weight/day, which corresponds to 37.1 g/day in a 70 kg adult. It is based on 3 human studies

(Elango et al., 2012; Pencharz et al., 2012; and Pencharz and Russell, 2012) and a rat study of Imamura et al. (2013). In a study of Pencharz et al. (2012), 5 young (25–35 y), healthy men received graded stepwise increases in intakes of 50, 150, 250, 500, 750, 1000, and 1250 mg/kg body weight/day; 7 dosages) which corresponded to the EAR and the EAR $\times$ 3,  $\times$ 5,  $\times$ 10,  $\times$ 15,  $\times$ 20, and  $\times$ 25 in a total of 29 studies. The endpoints were L-leucine oxidation and other surrogate markers such as plasma glucose, alanine aminotransferase, and ammonia to monitor for adverse effects. The metabolic limit to oxidize L-leucine was between 550 and 700 mg/kg body weight/day. The authors estimated the safe upper limit of L-leucine intake in humans and raises concerns that intakes over 550 mg/kg body weight/day or  $\sim$ 38.5 g/day may be a risk to health. The 90<sup>th</sup> percentile intakes of users under the intended use are far below this proposed upper limit of safe intake. Elango et al. (2012) aimed to determine the UL for L-leucine in adult men under acute dietary conditions in a study of 5 healthy adults (20-35 years) receiving graded stepwise increases in L-leucine intakes of 50, 150, 250, 500, 750, 1000, and 1250 mg/kg body weight/day, corresponding to the EAR and the EAR  $\times$ 3,  $\times$ 5,  $\times$ 10,  $\times$ 15,  $\times$ 20, and  $\times$ 25 in a total of 29 studies. The UL of L-leucine was identified by the measurement of plasma and urinary biochemical variables and changes in L-leucine oxidation by using L-[1-<sup>13</sup>C]-leucine. With L-leucine intakes  $>$ 500 mg/kg body weight/day, significant increases in blood ammonia concentrations above normal values, plasma L-leucine concentrations, and urinary L-leucine excretion were observed. A plateau in L-[1-<sup>13</sup>C]-leucine expressed as label tracer oxidation in breath was observed after 500 mg/kg/day, no clear plateau observed in L-leucine oxidation, and  $\alpha$ -ketoisocaproic acid oxidation appearing to plateau after 750 mg/kg/day. The authors suggested that, based on the plasma and urinary findings, an UL of L-leucine was 500 mg/kg/day or  $\sim$ 35 g/day in healthy adult men as a cautious estimate under acute dietary conditions. It is also supported by a rat study of Imamura et al. (2013) in which L-leucine intake up to 8% (or 5,300 mg/kg body weight/day) had no adverse effects except when animals were fed a low-protein diet. Considering that protein and energy requirements in rats are 10 times those of humans and considering that normal healthy people have moderate protein intakes and those practicing exercise or sport have generally high protein intakes, authors set an upper limit of safe intake of L-leucine in humans at 0.53 g/kg body weight/day. Overall, the body of evidence on oral administration of L-leucine in humans indicates that it can be consumed in considerable amounts without adverse effects.

Several human studies examining oral L-leucine supplementation on performance during exercise training or other outcomes reported no adverse effects of L-leucine in humans (Bjorkman et al., 2011; Casperson et al., 2012; Crowe et al., 2006; Ispoglou et al., 2011; Kirby et al., 2012; Leenders et al., 2011; Walker et al., 2010; Zemel et al., 2012). Doses tested were up to 12 g/day and the duration of the studies were up to 6 months. Our review also includes human clinical studies that were reported in GRN 308 (Swendseid et al., 1965; Mero et al., 1997, 2009; Pitkanen et al., 2003; Crowe et al., 2006; Verhoeven et al., 2009). The majority of the studies did not include assessment of safety-related endpoints, but rather focused on the effects of acute and prolonged dietary intake of L-leucine in individuals undergoing exercise training; however, the information in the human studies corroborates the safety information provided from animal studies.

In addition, no adverse effects were reported in associated with oral administration of up to 60 g/day of BCAAs providing 24 g/day L-leucine in healthy individuals (Gijsman et al., 2002;

Scarna et al., 2002) and bipolar subjects (Scarna et al., 2003). In patients with hepatic problems, supplementations of BCAA at daily doses of 28 g providing 11 g L-leucine/day for 1 year or 12-14 g BCAA providing 5.7 g L-leucine/day for 2-4 years were well tolerated with no adverse effects (Habu et al., 2009; Kawamura et al., 2009; Kobayashi et al., 2008; Kuroda et al., 2010; Marchesini et al., 2003; Poon et al., 2004; Yoshiji et al., 2002). Although these studies did not include assessment of safety-related endpoints, the information in the human studies corroborates the safety information provided from animal studies.

**POTENTIAL ADVERSE EFFECTS** (adopted from GRN 232 and IOM, 2005)

It appears, however, that the creation of imbalances among the BCAA (e.g., by dosing with high levels of any one of them) may sometimes induce reductions in appetite and growth (Block, 1989; Harper et al., 1984). However, these imbalances, which lead to catabolism of muscle, occur only in rats on marginally adequate protein diets (Block, 1989). The growth suppressing effects of L-leucine could be largely or fully prevented by the addition of amino acids (isoleucine, valine, phenylalanine, tryptophan, and threonine) or protein to the diet (Block, 1989). Harper et al. (1984) demonstrated that high dietary levels of L-leucine suppressed the growth of rats fed a low protein diet, and that the growth suppression could be prevented by supplementation with L-isoleucine and valine. There have been a number of attempts to study BCAA antagonisms in various tissues, and it appears that muscle is the major contributor to the depletion of L-isoleucine and L-valine pools in animals consuming high L-leucine diets. Other animal studies demonstrated a lack of adverse effects on growth when supplementary L-leucine was given at levels of up to approximately 6,000-8,000 mg/kg body weight/day or 10% L-leucine in diet, in conjunction with adequate dietary protein for periods ranging from 10 days to 13 weeks (Donato et al., 2006; Lynch et al., 2002; Sugawara et al., 2007; Tsubuku et al., 2004; Zhang et al., 2007). It is not at all clear that induced BCAA imbalances (except possibly in the case of animals on marginally adequate protein diets) have any adverse effects on growth (IOM, 2005).

Analysis of the NHANES dataset indicate that Americans' protein intake status is adequate and users of added L-leucine are likely to consume adequate levels of protein in the diet. Thus, L-leucine supplementation is not expected to cause a growth suppression problem or any other adverse effects in the American population.

GRN 308 (U.S. FDA, 2010) reported the results of the study by Verhoeven et al. (2009) in which the effects of 7.5 g/day L-leucine supplementation were tested on muscle mass and strength in 30 healthy elderly men (29 completed the study; n=15 in the L-leucine group and n=14 in the placebo group) for 3 months. No significant changes were reported in skeletal muscle mass or strength or in indexes of whole-body insulin sensitivity, blood glycated hemoglobin content, or the plasma lipid profile were observed. However, the fasting plasma L-valine levels of 15 healthy elderly men supplemented with 2.5 g of L-leucine at each main meal (i.e., 7.5 g/day) decreased by approximately 25% (vs. basal) within 2 weeks of treatment. The authors indicated that the clinical significance of this decline in plasma L-valine concentrations "remains debatable" because the plasma concentrations did not decline further after 2 weeks and remained within a normal physiologic range. Plasma L-isoleucine levels did not change significantly over the course of the study. No adverse effects were reported in this study. The dose of L-leucine (7.5 g) that resulted in a decrease in L-valine concentrations in this study is higher than the

## L-leucine GRAS

estimated intakes of L-leucine from the proposed food uses as EDI of all-users were 1.9 and 4.1 g at the mean and 90th percentile, respectively. The body of evidence on oral administration of L-leucine in humans indicates that it can be consumed in considerable amounts without adverse effects.

### **SAFETY DETERMINATION**

Numerous human and animal studies have reported the health benefits of L-leucine, an essential amino acid, with no major adverse effects. INNOBIO® uses a HACCP-controlled manufacturing process and rigorously tests its final production batches to verify adherence to quality control specifications. There is broad-based and widely disseminated knowledge concerning the chemistry of L-leucine. This GRAS determination is based on the data and information generally available and consented opinion about the safety of L-leucine. The literature indicates that L-leucine offers consumers health benefits without serious adverse effects.

The following safety evaluation fully considers the composition, intake, nutritional, microbiological, and toxicological properties of L-leucine as well as appropriate corroborative data.

1. The intended uses of L-leucine are the same as those described in GRN 308 and will substitute for currently marketed L-leucine products. L-Leucine is proposed for use as an ingredient in milk and non-milk based meal replacements, sports and isotonic beverages, vitamin enhanced waters, and meal replacement bars, at levels of 0.5-3.0 g/serving. Consequently, INNOBIO® notes that its uses will not result in any exposure beyond what was previously estimated. The proposed food uses result in an EDI for users (mean of 1.9 g/person/day or 28 mg/kg body weight/day and 90th percentile intake of 4.1 g/person/day or 64 mg/kg body weight/day) at levels significantly below those associated with any potential side effects.
2. In a 13-week rat toxicity study, the highest level studied for L-leucine product, 5% mixed into the diet, was established as the NOAEL. This NOAEL corresponded to a daily intake of 3,333 or 3,835 mg L-leucine/kg body weight/day for female and male rats, respectively, in addition to the amount provided by the basal diet. In a reproductive and developmental study, the highest dose administered by oral gavage, 1,000 mg/kg body weight/day, was established as the NOAEL for female reproductive function and embryo-fetal development. In addition, growth studies also showed that rats were fed diets supplemented with 10% L-leucine (approximately 8,200 mg/kg body weight/day) for 1-2 weeks had no statistically significant changes in body weight.
3. Human clinical studies reported no adverse effects associated with single oral supplementation of up to 60 g/day of BCAAs (providing 24 g L-leucine) in healthy subjects and bipolar subjects. Repeated dose studies showed that daily doses up to 12 g were well tolerated with no adverse effects in healthy subjects and subjects with type 2 diabetes. In patients with hepatic problems, long term supplementation of L-leucine at daily doses of 6-11g L-leucine (in 14-28 g BCAA) for 1 year and 5.7 g L-leucine (in 12 g

## L-leucine GRAS

BCAA/day) for 2-3 years were well tolerated with no adverse effects. Although these studies did not include assessment of safety-related endpoints, the information in the human studies corroborates the safety information provided from animal studies.

4. The IOM has not established the UL of protein or individual amino acids since for some well studied amino acids, there were no adverse effects reported at the highest dose tested in long term studies. The IOM also noted that many animal studies of amino acid toxicity were conducted with diets deficient in protein and that less emphasis was placed on these studies than those with adequate protein diets because of creation of amino acid imbalances. Recently, the ICAAS has proposed an upper limit of safe intake for L-leucine for healthy adults as 0.53 g/kg body weight/day, which may correspond to 37 g/day in a 70 kg adult. The NHANES analysis shows that Americans' protein intake status is considered adequate. Thus, BCAA antagonism would not be expected from the amount of L-leucine estimated to be consumed from the proposed food uses.
5. Authoritative government agencies including the U.S. FDA (2010) (GRN 308) have not raised questions regarding the safety of L-leucine as currently used in food. Since the FDA's last review of L-leucine in 2009-2010, no literature contradicting the agency's prior reviews/decisions has been published.
6. The compositional data and product properties are consistent with carefully controlled cGMP production and purification. L-leucine contains no impurities or contaminants of concern for human health. L-leucine is produced in accordance with cGMP and meets appropriate food-grade specifications. L-Leucine is obtained via fermentation of glucose and corn steep liquor using *Corynebacterium glutamicum* which has been safely used in industrial production of amino acids for a long time. INNOBIO® has established chemical and microbiological specifications consistent with other food-grade materials. Lot samples are routinely evaluated to verify compliance with the specifications.
7. Several reviews by experts in the field also have documented the safety of L-leucine.

**CONCLUSIONS AND GENERAL RECOGNITION OF THE SAFETY OF L-LEUCINE**

The intended use of L-leucine has been determined to be safe through scientific procedures as set forth in 21 CFR 170.3(b), thus satisfying the so-called “technical” element of the GRAS determination. In addition, because this safety evaluation was based on generally available and widely accepted data and information, it also satisfies the so-called “common knowledge” element of a GRAS determination.

On behalf of INNOBIO® Limited (INNOBIO®), we, the undersigned expert panel members, Susan Cho, Ph.D., Paul Pencharz, M.D., Ph.D., and Steven Heymsfield, M.D., have independently evaluated the materials summarized in the L-leucine Generally Recognized as Safe (GRAS) report. These individuals are qualified by scientific training and experience to evaluate the safety of substances intended to be added to foods. They have critically reviewed and evaluated the publicly available information summarized in this document and have individually and collectively concluded that L-leucine, produced consistent with cGMP and meeting the specifications described herein, is safe under its intended conditions of use. The Panel further unanimously concludes that these uses of L-leucine are GRAS based on scientific procedures and that other experts qualified to assess the safety of food and food ingredients would concur with these conclusions.

It is also INNOBIO’s opinion that the proposed use of L-leucine is not only safe within the terms of the Federal Food, Drug, and Cosmetic Act (meeting the standard of reasonable certainty of no harm), but it is also *Generally Recognized as Safe* (GRAS) according to Title 21 Code of Federal Regulations (21 CFR) according to consensus among experts.

\_\_\_\_\_  
Susan Cho, Ph.D.  
NutraSource, Inc.

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Date

\_\_\_\_\_  
Paul Pencharz, M.D., Ph.D.  
The University of Toronto

\_\_\_\_\_  
Date

\_\_\_\_\_  
Steven Heymsfield, M.D.  
Pennington Biomedical Research Center

\_\_\_\_\_  
Date

**CONCLUSIONS AND GENERAL RECOGNITION OF THE SAFETY OF L-LEUCINE**

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(b) (6)

Susan Cho, Ph.D.

NutraSource, Inc. *✓*

4/7/2014  
Date

(b) (6)

Paul Pencharz, M.D., Ph.D. *✓*

The University of Toronto

April 6<sup>th</sup> 2004  
Date

(b) (6)

Steven Heymsfield, M.D.

Pennington Biomedical Research Center

4/2/2014  
Date

## REFERENCES

Björkman MP, Pilvi TK, Kekkonen RA, Korpela R, Tilvis RS. Similar effects of L-leucine rich and regular dairy products on muscle mass and functions of older polymyalgia rheumatica patients: a randomized crossover trial. *J Nutr Health Aging*. 2011;15:462-467.

Block KP, Harper AE. L-valine metabolism in vivo. effects of high dietary levels of L-leucine and isoleucine. *Metabolism*. 1984;33:559-566.

Casperson SL, Sheffield-Moore M, Hewlings SJ, Paddon-Jones D. L-leucine supplementation chronically improves muscle protein synthesis in older adults consuming the RDA for protein. *Clin Nutr*. 2012;31:512-519.

CDC. 2006. *Analytical and Reporting Guidelines. The National Health and Nutrition Examination Survey (NHANES)*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: [http://www.cdc.gov/nchs/data/nhanes/nhan0e3s\\_04/nhanes\\_analytic\\_guidelines\\_dec2005.pdf](http://www.cdc.gov/nchs/data/nhanes/nhan0e3s_04/nhanes_analytic_guidelines_dec2005.pdf).

Chen H, Simar D, Ting JH, Erkelens JR, Morris MJ. L-leucine improves glucose and lipid status in offspring from obese dams, dependent on diet type, but not caloric intake. *J Neuroendocrinol*. 2012;24:1356-1364.

Crowe MJ, Weatherson JN, Bowden BF. Effects of dietary L-leucine supplementation on exercise performance. *Eur J Appl Physiol*. 2006;97:664-672.

Cynober L, Bier DM, Kadowaki M, Morris SM Jr., Renwick AG. A proposal for an upper limit of L-leucine safe intake in healthy adults. *J Nutr*. 2012;142:2249S-2250S.

Donato J Jr, Pedrosa RG, Cruzat VF, Pires IS, Tirapegui J. Effects of L-leucine supplementation on the body composition and protein status of rats submitted to food restriction. *Nutrition*. 2006;22:520-527.

Elango R, Chapman K, Rafii M, Ball RO, Pencharz PB. Determination of the tolerable upper intake level of L-leucine in acute dietary studies in young men. *Am J Clin Nutr*. 2012;96:759-767.

Gijssman HJ, Scarnà A, Harmer CJ, McTavish SB, Odontiadis J, Cowen PJ, Goodwin GM. A dose-finding study on the effects of branch chain amino acids on surrogate markers of brain dopamine function. *Psychopharmacology (Berl)*. 2002;160:192-197.

Habu D, Nishiguchi S, Nakatani S, Lee C, Enomoto M, Tamori A, Takeda T, Ohfuji S, Fukushima W, Tanaka T, Kawamura E, Shiomi S. Comparison of the effect of BCAA granules on between decompensated and compensated cirrhosis. *Hepatogastroenterology*. 2009;56:1719-1723.

## L-leucine GRAS

Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. *Annu Rev Nutr.* 1984;4:409-454.

Imamura W, Yoshimura R, Takai M, Yamamura J, Kanamoto R, Kato H. Adverse effects of excessive L-leucine intake depend on dietary protein intake: a transcriptomic analysis to identify useful biomarkers. *J Nutr Sci Vitaminol (Tokyo).* 2013;59:45-55.

IOM. 2005. Protein and Amino Acids. In: *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients)*. Washington, DC: Institute of Medicine (IOM), National Academy Press (NAP), pp. 589-768. Available at: <http://www.nap.edu/openbook.php?recordid=104908page=589>

Ispoglou T, King RF, Polman RC, Zanker C. Daily L-leucine supplementation in novice trainees during a 12-week weight training program. *Int J Sports Physiol Perform.* 2011;6:38-50.

Kawamura E, Habu D, Morikawa H, Enomoto M, Kawabe J, Tamori A, Sakaguchi H, Saeki S, Kawada N, Shiomi S. A randomized pilot trial of oral branched-chain amino acids in early cirrhosis: validation using prognostic markers for pre-liver transplant status. *Liver Transpl.* 2009;15:790-797.

Kirby TJ, Triplett NT, Haines TL, Skinner JW, Fairbrother KR, McBride JM. Effect of L-leucine supplementation on indices of muscle damage following drop jumps and resistance exercise. *Amino Acids.* 2012;42:1987-96.

Kobayashi M, Ikeda K, Arase Y, Suzuki Y, Suzuki F, Akuta N, Hosaka T, Murashima N, Saitoh S, Someya T, Tsubota A, Kumada H. Inhibitory effect of branched-chain amino acid granules on progression of compensated liver cirrhosis due to hepatitis C virus. *J Gastroenterol.* 2008;43:63-70.

Kuroda H, Ushio A, Miyamoto Y, Sawara K, Oikawa K, Kasai K, Endo R, Takikawa Y, Kato A, Suzuki K. Effects of branched-chain amino acid-enriched nutrient for patients with hepatocellular carcinoma following radiofrequency ablation: a one-year prospective trial. *J Gastroenterol Hepatol.* 2010;25:1550-1555.

Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Hartgens F, Wodzig WK, Saris WH, van Loon LJ. Prolonged L-leucine supplementation does not augment muscle mass or affect glycemic control in elderly type 2 diabetic men. *J Nutr.* 2011;141:1070-1076.

Lynch CJ, Hutson SM, Patson BJ, Vaval A, Vary TC. Tissue-specific effects of chronic dietary L-leucine and norleucine supplementation on protein synthesis in rats. *Am J Physiol Endocrinol Metab.* 2002;283:E824-E835.

Marchesini G, Bianchi G, Merli M, Amodio P, Panella C, Loguercio C, Rossi Fanelli F, Abbiati R; Italian BCAA Study Group. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. *Gastroenterology.* 2003;124:1792-1801.

## L-leucine GRAS

Matsuzaki K, Kato H, Sakai R, Toue S, Amao M, Kimura T. Transcriptomics and metabolomics of dietary L-leucine excess. *J Nutr.* 2005;13546, Suppl:1571 S-I 5755.

Mawatari K, Katsumata T, Uematsu M, Katsumata T, Yoshida J, Smriga M, Kimura T. Prolonged oral treatment with an essential amino acid L-leucine does not affect female reproductive function and embryo-fetal development in rats. *Food Chem Toxicol.* 2004;42:1505-1511.

Mero A, Pitkänen H, Oja SS, Komi PV, Pöntinen P, Takala T. L-leucine supplementation and serum amino acids, testosterone, cortisol and growth hormone in male power athletes during training. *J Sports Med Phys Fitness.* 1997;37:137-145.

Mero A, Leikas A, Knuutinen J, Hulmi JJ, Kovanen V. Effect of strength training session on plasma amino acid concentration following oral ingestion of leucine, BCAAs or glutamine in men. *Eur J Appl Physiol.* 2009;105:215-223.

Millward DJ. Knowledge gained from studies of L-leucine consumption in animals and humans. *J Nutr.* 2012;142:2212S-2219S.

Pencharz PB, Russell RM. Application of Key Events Dose Response Framework to defining the upper intake level of leucine in young men. *J Nutr.* 2012;142:2225S-2226S.

Pencharz PB, Elango R, Ball RO. Determination of the tolerable upper intake level of leucine in adult men. *J Nutr.* 2012;142:2220S-2224S.

Pitkänen HT, Oja SS, Rusko H, Nummela A, Komi PV, Saransaari P, Takala T, Mero AA. L-leucine supplementation does not enhance acute strength or running performance but affects serum amino acid concentration. *Amino Acids.* 2003 Jul;25:85-94.

Poon RT, Yu WC, Fan ST, Wong J. Long-term oral branched chain amino acids in patients undergoing chemoembolization for hepatocellular carcinoma: a randomized trial. *Aliment Pharmacol Ther.* 2004;19:779-788.

Sargentini NJ, Smith KC. Mutagenesis by normal metabolites in *Escherichia coli* phenylalanine mutagenesis is dependent on error-prone DNA repair. *Mutat Res.* 1986;161:113-118.

Scarnà A, Gijsman HJ, Harmer CJ, Goodwin GM, Cowen PJ. Effect of branch chain amino acids supplemented with tryptophan on tyrosine availability and plasma prolactin. *Psychopharmacol. (Berl).* 2002;159:222-223.

Scarna A, Gijsman HJ, McTavish SF, Harmer CJ, Cowen PJ, Goodwin GM. Effects of a branched-chain amino acid drink in mania. *Br J Psychiatry.* 2003;182:210-213.

Sugawara T, Ito Y, Nishizawa N, Nagasawa T. Supplementation with dietary L-leucine to a protein-deficient diet suppresses myofibrillar protein degradation in rats. *J Nutr Sci Vitaminol.* 2007;53:552-555.

## L-leucine GRAS

Swendseid ME, Villalobos J, Figueroa WS, Drenick EJ. The effects of test doses of leucine, L-isoleucine or L-valine on plasma amino acid levels. The unique effect of L-leucine *Am J Clin Nutr.* 1965;17:317-321.

Tsubuku S, Hatayama K, Katsumata T, Nishimura N, Mawatari K, Smriga M et al. Thirteen-week oral toxicity study of branched-chain amino acids in rats. *Int J Toxicol.* 2004;23:119-126.

U.S. FDA. 2010. Agency Response Letter GRAS Notice No. GRN 000308 (L-leucine). College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN).

U.S. FDA. 2009. U.S. Code of Federal Regulations (CFR). Title 21-Food and Drugs (Food and Drug Administration). Washington (DC): U.S. Government Printing Office (GPO). Available at: <http://www.access.gpo.gov/cqi-bin/cfr/asmblclqe/i?title=200821> [See Table for CFR sections used].

USDA. 2006. USDA National Nutrient Database for Standard Reference. Release 19. Available at: <http://www.ars.usda.gov/Services/docs.htm?docid=8964>.

Verhoeven S, Vanschoonbeek K, Verdijk LB, Koopman R, Wodzig WK, Dendale P et al. Long-term L-leucine supplementation does not increase muscle mass or strength in healthy elderly men. *Am J Clin Nutr.* 2009; 89:1468-1475.

Walker TB, Smith J, Herrera M, Lebegue B, Pinchak A, Fischer J. The influence of 8 weeks of whey-protein and L-leucine supplementation on physical and cognitive performance. *Int J Sport Nutr Exerc Metab.* 2010;20:409-417.

Witham WG, Yester KA, McGaffin KR. A high L-leucine diet mitigates cardiac injury and improves survival after acute myocardial infarction. *Metabolism.* 2013;62:290-302.

Xing W, Na R. Amino acids excess increase SCEs in human lymphocytes. *Mutat Res.* 1996;372:75-78.

Yoshiji H, Noguchi R, Ikenaka Y, Kaji K, Aihara Y, Douhara A, Yamao J, Toyohara M, Mitoro A, Sawai M, Yoshida M, Morioka C, Fujimoto M, Uemura M, Fukui H. Combination of branched-chain amino acid and angiotensin-converting enzyme inhibitor improves liver fibrosis progression in patients with cirrhosis. *Mol Med Rep.* 2012;5:539-544.

Zemel MB, Bruckbauer A. Effects of a L-leucine and pyridoxine-containing nutraceutical on fat oxidation, and oxidative and inflammatory stress in overweight and obese subjects. *Nutrients.* 2012;4:529-541.

Zhang Y, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH. Increasing dietary L-leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. *Diabetes.* 2007; 56:1647-1654.

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**Appendix C. Certificate of Analysis**

INNOBIO® Instantized L-Leucine Powder

Code: (b) (4)

Lot No.:

Date of Manufacture: 08/25/2013

Description: INNOBIO® Instantized L-Leucine Powder is made using a patented micro-encapsulation process and it contains 0.5% Lecithin. The L-leucine is from fermentation.

Tests	Limits	Methods	Results
Appearance	White to off-white fine powder	Visual	Conforms
L-Leucine Content (%)	98.0 Min.	HPLC	98.8
Bulk Density (g/ml)	0.30-0.60	USP 616	0.54
Particle Size	95% through 40 mesh	USP 786	99.5
Loss on Drying (%)	1.0 Max.	USP 731	0.16
Residue on Ignition (%)	0.40 Max.	USP 281	0.13
Lead (ppm)	1.0 Max.	EN ISO 17294	< 0.050
Arsenic (ppm)	1.0 Max.	EN ISO 17294	< 0.050
Cadmium (ppm)	1.0 Max.	EN ISO 17294	< 0.01
Mercury (ppm)	0.1 Max.	BS EN 13806	0.06
Total Plate Count (CFU/g)	3,000 Max.	ISO 4833	130
Yeasts and Molds (CFU/g)	100 Max.	ISO 7954	40
<i>Coliform</i> (CFU/g)	10 Max.	ISO 4832	< 10
<i>E. coli</i>	Negative	ISO 16649	Negative
<i>Salmonella</i>	Negative/25g	ISO 6579	Negative
<i>Staphylococcus aureus</i>	Negative/g	ISO 6888	Negative

Packaging: Packaged using a double layer polyethylene bag liner in a cardboard drum, 25 kg net.

Storage and Handling: Store in a cool dry place isolated from strong odors.

Shelf Life: Two years in the original unopened package. It is recommended to use the entire contents after opening.

Approval: (b) (6)

Date: 09/05/2013

L-leucine GRAS

INNOBIO® Instantized L-Leucine Powder

Code: (b) (4)

Lot No.: (b) (4)

Date of Manufacture: 05/15/2012

Description: INNOBIO® Instantized L-Leucine Powder is made with a patented micro-encapsulation process and it contains 0.5% Lecithin. The L-leucine is from fermentation.

Tests	Limits	Methods	Results
Appearance	White to off-white fine powder	Visual	Conforms
L-Leucine Content (%)	98.0 Min.	HPLC	99.4
Bulk Density (g/ml)	0.30-0.60	USP 616	0.46
Particle Size	95% through 40 mesh	USP 786	98.8
Loss on Drying (%)	1.0 Max.	USP 731	0.02
Residue on Ignition (%)	0.40 Max.	USP 281	0.25
Lead (ppm)	1.0 Max.	EN ISO 17294	< 0.050
Arsenic (ppm)	1.0 Max.	EN ISO 17294	< 0.050
Cadmium (ppm)	1.0 Max.	EN ISO 17294	< 0.01
Mercury (ppm)	0.1 Max.	BS EN 13806	< 0.005
Total Plate Count (CFU/g)	3,000 Max.	ISO 4833	205
Yeasts and Molds (CFU/g)	100 Max.	ISO 7954	45
<i>Coliform</i> (CFU/g)	10 Max.	ISO 4832	< 10
<i>E. coli</i>	Negative	ISO 16649	Negative
<i>Salmonella</i>	Negative/25g	ISO 6579	Negative
<i>Staphylococcus aureus</i>	Negative/g	ISO 6888	Negative

Packaging: Packaged using a double layer polyethylene bag liner in a cardboard drum, 25 kg net.

Storage and Handling: Store in a cool dry place isolated from strong odors.

Shelf Life: Two years in the original unopened package. It is recommended to use the entire contents after opening.

Approval: (b) (6)

Date: 05/30/2012

L-leucine GRAS

INNOBIO® Instantized L-Leucine Powder

Code: (b) (4)

Lot No.: (b) (4)

Date of Manufacture: 05/19/2012

Description: INNOBIO® Instantized L-Leucine Powder is made with a patented micro-encapsulation process and it contains 0.5% Lecithin. The L-leucine is from fermentation.

Tests	Limits	Methods	Results
Appearance	White to off-white fine powder	Visual	Conforms
L-Leucine Content (%)	98.0 Min.	HPLC	99.5
Bulk Density (g/ml)	0.30-0.60	USP 616	0.49
Particle Size	95% through 40 mesh	USP 786	98.9
Loss on Drying (%)	1.0 Max.	USP 731	0.01
Residue on Ignition (%)	0.40 Max.	USP 281	0.11
Lead (ppm)	1.0 Max.	EN ISO 17294	< 0.050
Arsenic (ppm)	1.0 Max.	EN ISO 17294	< 0.050
Cadmium (ppm)	1.0 Max.	EN ISO 17294	< 0.01
Mercury (ppm)	0.1 Max.	BS EN 13806	< 0.005
Total Plate Count (CFU/g)	3,000 Max.	ISO 4833	90
Yeasts and Molds (CFU/g)	100 Max.	ISO 7954	20
<i>Coliform</i> (CFU/g)	10 Max.	ISO 4832	< 10
<i>E. coli</i>	Negative	ISO 16649	Negative
<i>Salmonella</i>	Negative/25g	ISO 6579	Negative
<i>Staphylococcus aureus</i>	Negative/g	ISO 6888	Negative

Packaging: Packaged using a double layer polyethylene bag liner in a cardboard drum, 25 kg net.

Storage and Handling: Store in a cool dry place isolated from strong odors.

Shelf Life: Two years in the original unopened package. It is recommended to use the entire contents after opening.

Approval: (b) (6)

Date: 05/31/2012

Pages 000074-000225 have been removed in accordance with copyright laws. Please see the bibliography list of the references that have been removed from this request on pages 000058-000061.

**SUBMISSION END**