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ORIGINAL SUBMISSION

JHeimbach LLC

RECD JUL 31 2006

July 26, 2006

Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Premarket Approval
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Sir:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997),Taiyo International, through me as its agent, herby provides notice of a claim that the food ingredient described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS), based on scientific procedures, for addition to foods as described to provide consumers with a supplementary dietary source of the amino acid theanine.

As required, three copies of the notification are provided.

If you have any questions regarding this notification, please feel free to contact me at .

Sincerely,

James T. Heimbach, Ph.D., F.A.C.N.
President

4530 Broad Branch Road, N.W., Washington, DC 20008 USA
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I. GRAS Exemption Claim

Taiyo International, Inc., through its agent JHeimbach LLC, hereby notifies the Food and Drug Administration that the use of Suntheanine® L-theanine described below is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Taiyo International has determined that such use is generally recognized as safe (GRAS) through scientific procedures.

James T. Heimbach, Ph.D., F.A.C.N.
President, JHeimbach LLC

7/26/06
Date

A. Name and Address of Notifier

Taiyo International, Inc.
5960 Golden Hills Drive
Minneapolis, MN 55416

Contact: Scott J. Smith
Telephone:
Facsimile:
E-mail:

B. Name of GRAS Substance

The common name of the substance that is the subject of this GRAS notice is Suntheanine®. This is a branded product that is entirely composed of the amino acid L-theanine (N-ethyl-L-glutamine or L-glutamic acid-γ-monoethylamide).

C. Intended Use and Consumer Exposure

Suntheanine is intended to be added to foods in a number of different food categories to provide up to 250 mg/serving of L-theanine. The food categories in which use of Suntheanine is intended include fruit juices and drinks, non-herbal teas, sports beverages, specialty bottled waters, chocolate bars and chews, hard candies and breath mints, and chewing gum. Over 85% of Americans age 2 or older are users of at least one of these categories of food, and the mean estimated daily intake of Suntheanine from these intended uses is 628 mg/day, equivalent to 11.3 mg/kg bw/day. The estimated daily intake (EDI), corresponding to the 90th percentile of intake of Suntheanine, is 1284 mg/day, equivalent to 24.2 mg/kg bw/day.

D. Basis for GRAS Determination

Taiyo's GRAS determination for the intended uses of Suntheanine is based on scientific procedures as described under 21 CFR §170.30(b). A comprehensive search of the literature through June 2006 was conducted by JHeimbach LLC and served as the basis for preparation of a monograph summarizing the totality of the information available germane to determining the safety of the intended uses of Suntheanine.

Tea is the only naturally occurring source of theanine in the diets of Americans and, in fact, of most people of the world. After water, tea is the most widely consumed beverage in the world. It was estimated in 2003 that per capita consumption of tea worldwide was about 120 ml/day, approximately 77% black tea, 21% green tea, and 2% oolong tea. The Food and Agricultural Organization (FAO) estimated that world production of tea in 2000 was 2.8 million metric tons and projected that it would reach 3.3 metric tons by 2010. FAO also published data showing that tea consumption in many other countries is considerably higher than in the United States; it is more than 8 times higher in Ireland, for example, and nearly 4 times higher in Japan. Even in the United States, where tea competes with coffee and numerous other beverages, it is consumed by many adults and children. In the 1999–2002 National Health and Nutrition Examination Survey (NHANES), about 20% of adult respondents reported having consumed tea on the survey day. The mean amount of tea consumed by these individuals was 763 ml, but the top 10% of consumers drank 1650 ml of tea and the top 1% of consumers drank 3337 ml.

The theanine content of tea leaves is generally in the range of 1–2.5%; since theanine is highly soluble in water, nearly all of the theanine in the leaves is likely to dissolve in the water when a tea beverage is prepared. The estimated intake of theanine—nearly all of it L-theanine—by tea drinkers in the United States was estimated to be 152–382 mg/day at the mean, and as much as 667–1668 mg at the 99th percentile, these ranges reflecting the range of theanine content in tea leaves. This last range, although it is still lower than is found in some parts of the world, encompasses the level of theanine intake estimated to result from the intended uses of Suntheanine.

Metabolism and toxicokinetic studies have shown that the intestinal absorption of L-theanine is mediated by a Na^+-coupled co-transporter in the brush-border membrane. After absorption, theanine is rapidly incorporated into the blood and many tissues such as the liver and brain, this last via a leucine-preferring transport system. Theanine is hydrolyzed to glutamine and ethylamine, predominantly in the kidneys. Much of both metabolites is immediately excreted, but some returns to the plasma. Both theanine and its metabolites reach peak concentrations in the plasma and in tissues within a few hours, and then rapidly decrease along with a concomitant increase in urinary concentrations. The evidence indicates that theanine does not accumulate in plasma but is rapidly excreted.

The oral LD50 of Suntheanine is >5000 mg/kg, showing a low order of oral toxicity. A subacute (28-day) study obtained an oral NOAEL at the only dose tested, 2000 mg/kg bw/day. In a 13-week subchronic dietary study, rats were dosed with 1500, 3000, or 4000 mg Suntheanine/kg bw/day. No toxicity was found at any tested dose, and the NOAEL was the

highest dose tested, 4000 mg/kg bw/day. A 78-week chronic study of Suntheanine in mice found no evidence of carcinogenicity and genetic assays have shown that it is not mutagenic.

An Expert Panel determined the intended use of Suntheanine® to be safe, and also GRAS, by demonstrating that the safety of this level of intake is based on publicly available and accepted information and is generally recognized by experts qualified by scientific training and experience to evaluate the safety of substances added to food. Determination of the safety and GRAS status of Suntheanine for direct addition to food under its intended conditions of use was made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., Robert J. Nicolosi, Ph.D., and Michael W. Pariza, Ph.D., who reviewed the information in this monograph as well as other information available to them. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. They critically reviewed and evaluated the publicly available information, including the potential human exposure to Suntheanine resulting from its intended uses, and individually and collectively concluded that the available information on Suntheanine contains no evidence that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public health when Suntheanine is used under its intended conditions of use.

It is the Expert Panel's opinion that other qualified scientists reviewing the same publicly available data would reach the same conclusion. Therefore, Suntheanine is GRAS by scientific procedures under the conditions of use described.

E. Availability of Information

The data and information that serve as the basis for the GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of James T. Heimbach, Ph.D., President, JHEIMBACH LLC, 4530 Broad Branch Road NW, Washington DC 20008,

II. IDENTITY OF THE SUBSTANCE

A. Chemical Name

The product that is the subject of this GRAS notice is Suntheanine® brand L-theanine, a nutrient supplement marketed by Taiyo International, Inc. The systemic chemical names of L-theanine are N-ethyl-L-glutamine and L-glutamic acid-γ-monoethylamide.

B. Trade or Common Name

The trade name of the product is Suntheanine®.

C. CAS Registry Numbers

The Chemical Abstracts Service (CAS) registry number for L-theanine is 3081-61-6.

D. Molecular and Structural Formula

The molecular formula of L-theanine is $C_7H_{14}N_2O_3$. The molecular weight is 174.20. The structural formula is shown in Figure 1.

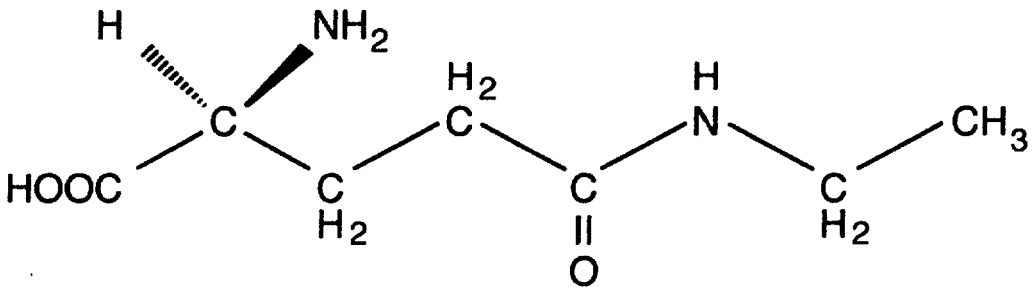

Figure 1. Structural Formula of L-Theanine

E. Physical and Chemical Properties

A brief summary of the physical and chemical properties of Suntheanine is provided in Table 1.

Table 1. Physical and Chemical Properties of Suntheanine

Physical/Chemical Property	Value
Appearance	White crystalline powder
Appearance in solution	Transparent, colorless
Taste	Slightly sweet
Odor	None
Melting point	214–215°C
Water solubility	Soluble
Ethanol solubility	Insoluble
Ether solubility	Insoluble

F. Production Process: Enzymatic Synthesis

L-theanine occurs naturally in tea (*Camellia sinensis*) and in one species of mushroom, *Xerocomus badius* (Graham 1992, Ekborg-Ott et al. 1997), which is not edible. However, the theanine in tea is a mixture of the L-theanine and D-theanine enantiomers, with D-theanine constituting an average of 1.85% of the total theanine in 17 types of tea tested (Ekborg-Ott et al. 1997). As demonstrated in the next section, Suntheanine, produced by enzymatic synthesis, contains no detectible D-theanine.

The starting materials for the manufacture of Suntheanine are food-grade L-glutamine and ethylamine, the same substances that are the precursors in the biosynthesis of theanine in the root of the tea plant (Ekborg-Ott et al. 1997). As shown in the schematic diagram in Figure 2, the synthesis of Suntheanine is accomplished enzymatically.

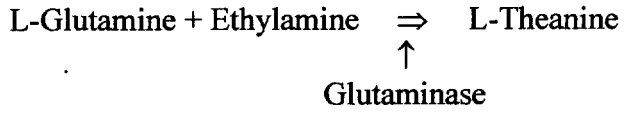

Figure 2. Schematic of Enzymatic Synthesis of Suntheanine® Brand of L-Theanine

The enzyme used in the synthesis of Suntheanine is glutaminase produced by either of two microorganisms. The first is *Pseudomonas nitroreducens*, developed by Iizuka and Komagata (1964) and identified by the Institute for Fermentation, Osaka, Japan, as IFO 12694 and by Ajinomoto Co. Inc., Kawasaki, Japan, as AJ2282. The American Type Culture Collection (ATCC) designation of this bacterium is ATCC 33634. The second is *Bacillus amyloliquefaciens*, a member of the heterogeneous *Bacillus subtilis* group, first published in 1987 (Priest et

al. 1987). The *B. amyloliquefaciens* strain intended for use as an alternate source of glutaminase is designated GT2. In 16S ribosomal nucleotide sequence analysis, it fully matches *B. subtilis* strain ATCC 9799.

In preparation for production, the organism is aerobically cultured in a medium of glucose and yeast extract with sodium glutamate, ethylamine hydrochloride, K_2HPO_4, KH_2PO_4, EDTA-Fe, and$MgSO_4 \bullet 7H_2O$. Cells are collected by centrifugation and washed with saline solution.

The washed cells are suspended in water containing a small amount of NaCl; κ-Carrageenan is dissolved in water and the two solutions are mixed and cooled to form a gel. The gel is soaked in a KCl solution at to increase gel strength. The resulting gel is granulated, washed with a KCl solution and suspended in a phosphate buffer containing KCl and hexamethylenediamine. The mixture is shaken, filtered, and washed with a KCl solution. The cells thus immobilized are suspended in an acetate buffer containing glutaraldehyde. The resulting immobilized cells are thoroughly washed with deionized water before use.

The reactor consists of four cm columns, each containing both immobilized cells and fresh wet cells, connected in parallel; two vessels for glutamine and ethylamine solutions; a pump; and a product solution reservoir. The pH levels of the glutamine and ethylamine solutions are adjusted as required with borate-NaOH buffer. The flow rate of the substrate solution in the reactor system is carefully controlled. The solution containing glutamine and ethylamine is supplied through the inlet.

The reacted mixture is cooled and the precipitate removed by filtration. The supernatant mixture is concentrated and dried under reduced pressure and the precipitate dissolved in hot ethanol and crystallized by cooling. The resulting crystals are again filtered and re-crystallized in cold ethanol. Theanine is obtained as white crystals. The crystals are collected by centrifugation and dried in a fluidized bed dryer.

Glutamine, ethylamine, and glutamic acid derived from raw materials, as well as ammonia produced during the reaction, are potential impurities. Ammonia gas is released during the production process. The remaining components, if present, would be detected by the HPLC assay methods discussed in the next section.

G. Product Characteristics

1. Identification of Suntheanine with Theanine

a. Spectrometric Analysis

Samples from three lots of Suntheanine (Lots 805251, 703181, 606101) were analyzed by spectrometric methods to identify its chemical structure and to confirm its chemical equivalency to theanine. Lot C040097I088F of L-theanine from Funakoshi Chemicals Co., Ltd., Japan, sold as Theanine R, was used as the reference chemical reagent, and its purity was specified at more than 97%.

(1) Mass Spectrometry

The mass spectra of Suntheanine and theanine (Theanine R) dissolved in glycerin were compared. Both Suntheanine and Theanine R reveal a molecular ion peak at *m/e* 175, indicating molecular weights of 174. Additional peak signals at *m/e* 93 and 185 were considered as peaks of glycerol and diglycerol, respectively, from the glycerin in which the samples were dissolved. No other peaks were observed around the two peaks at *m/e* 175 and 185.

Data obtained in this study indicate that Suntheanine has a molecular weight of 174, which matches that of Theanine R (theanine). This result demonstrates that the molecular weight of Suntheanine is identical to that of theanine.

(2) Infrared (IR) Spectrometry

Samples of Suntheanine and theanine (Theanine R) were tableted with KBr and exposed to infrared radiation in the range of 10000–100 cm^{-1} (1–100 μm), which is absorbed and converted by organic molecules into energy of molecular vibration.

The IR spectrum of Suntheanine showed characteristic absorption peaks in the range of 3400–2000 cm^{-1} for the amino group and in the range of 1700–1400 cm^{-1} for the carbonyl, amide and amino groups. The Fourier transform-infrared spectra of Suntheanine and Theanine R were overlapped by FIR instrumentation, and comparison of the two spectra indicated that these two substances were chemically identical.

(3) Proton Nuclear Magnetic Resonance (NMR) Spectrometry

The chemical structure of organic compounds is revealed by NMR analysis. By measuring frequency shifts from a reference marker, an accurate count of protons (hydrogen atoms) can be detected from accumulated peak areas. Spectra of Suntheanine and theanine (Theanine R) showed the same peaks at δ (ppm) = 1.16 (3H at $NHCH_2\mathit{CH_3}$), 2.18 (2H at $CH\mathit{CH_2}CH_2COO$), 2.44 (2H at $CHCH_2\mathit{CH_2}COO$), 3.25 (2H at $NH\mathit{CH_2}CH_3$), and 3.81 (1H at $\mathit{CH}CH_2CH_2CH_2COO$), respectively. A signal peak of acetone as a reference marker was observed at 2.22. These data strongly suggest that Suntheanine is identical to theanine in chemical structure.

(4) Conclusion from Spectrometric Analyses

The above three spectrometric analyses demonstrate the chemical equivalency of Suntheanine and theanine (Theanine R) with respect to both molecular weight and chemical structure. Together, these analyses prove that Suntheanine is indeed theanine.

b. High Performance Liquid Chromatography (HPLC) Analysis

Five lots of Suntheanine were tested three times, along with samples of Theanine R, using L-norleucine as an internal standard for comparison. The mean of the peak area in the Suntheanine chromatograms corresponded to that of Theanine R in each sample, and the ratio of the peak area of the Suntheanine solution to that of L-norleucine matched the ratio for Theanine R, as shown in Table 2.

Table 2. Peak Areas of Suntheanine and Theanine R and Ratios of Theanine/Norleucine

Sample	Suntheanine					Theanine R
Lot	[redacted]					
Concentration (ppm)	10	10	10	10	10	10
Area Peaks of Suntheanine and Theanine R						
No. 1	13529	13268	14164	14050	14330	13861
No. 2	14177	13969	14393	14358	15320	14023
No. 3	14322	14381	14852	15461	16134	14240
Average Area Peaks	14009	13873	14470	14623	15261	14041
Area Peaks of Norleucine						
No. 1	9367	8610	9397	8986	9322	10097
No. 2	9918	9204	9545	9323	9544	10118
No. 3	10097	9412	9707	9562	9793	10206
Ratio of Area Peaks						
No. 1	1.44428	1.54101	1.50738	1.56349	1.53727	1.37272
No. 2	1.42949	1.51781	1.50782	1.53995	1.60515	1.38599
No. 3	1.41845	1.52791	1.53001	1.61687	1.64745	1.39528
Average Ratio of Area Peaks	1.43074	1.52891	1.51507	1.57344	1.59662	1.38466

The HPLC results confirm the findings of the spectrometric analyses in identifying Suntheanine and theanine as being chemically identical.

2. Enantiomeric Identity

As was noted earlier, naturally occurring theanine is a mixture comprising two enantiomers, L-theanine and D-theanine, with the L- enantiomeric form predominant. Suntheanine, however, does not contain detectible levels of the D- enantiomer.

Desai and Armstrong (2004) describe a HPLC methodology, based on derivatizing theanine with 9-fluorenylmethyloxycarbonyl chloride, to separate theanine enantiomers. The method was used to assess the enantiomeric composition of a variety of types of tea. When the method was applied to Suntheanine, no D-theanine was detected (Desai and Armstrong 2004), although it was in five other commercially available theanine products (which, indeed, appeared to be essentially racemic).

Desai and Armstrong (2004) did not report the limit of detection (LOD) for D-theanine in a predominantly L-theanine mixture, but more recently Armstrong, in an unpublished and undated manuscript, speculated that the method may not provide adequate separation to detect trace levels of D-theanine. For this reason, Armstrong developed a new HPLC method that provides improved separation and is claimed to be capable of detecting 0.1% D-theanine in a sample comprising 99.9% L-theanine. The new method was used to analyze a number of samples of Suntheanine, and again no D-theanine was seen. Armstrong thus concluded that Suntheanine is greater than 99.9% L-theanine.

3. Purity

a. Glutaminase Residues

Since Suntheanine is produced by a fermentation method mediated by glutaminase, analyses were conducted to assure that no residual glutaminase remains in the final product. Two approaches were used to test for the presence of glutaminase in Lot 109041 of Suntheanine, an enzyme-activity test and a test for proteinaceous material.

Enzyme activity was measured by the formation of *p*-nitroaniline in a reaction mixture containing Suntheanine along with γ-glutamyl *p*-nitroanilide and imidazole-HCl buffer (pH 9.0). The enzyme reaction was stopped by adding acetic acid to the reaction mixture. *p*-nitroaniline was estimated from the absorbance at 410 nm. One unit of the enzyme activity was defined as the amount which forms 1 μmole of *p*-nitroaniline per minute at 30°C.

Reference standards were created by culturing one of the production organisms, *Pseudomonas nitroreducens*, and disrupting the cells for 2 minutes using an ultrasonic oscillator. Supernatant obtained by centrifugation was added to a 33% saturated ammonium sulfate solution. The precipitate and the supernatant were collected by centrifugation. Reference standards included the cells themselves, the supernatant, and the precipitate. The results are shown in Table 3. It can be seen that no enzyme activity was evident in the Suntheanine solution, although the methodology was successful in detecting it in the reference standards. The protein concentration was measured by Lowry's method.

Table 3. Enzyme Activity of Suntheanine and Reference Standards

Sample	Enzyme Activity (U/mg)
20% Suntheanine solution	0
P. nitroreducens microorganism cell	0.380
Supernatant of ammonium sulfate saturation	0.737
Precipitate of ammonium sulfate saturation	0.097

To confirm the absence of glutaminase in Suntheanine, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to test for the presence of protein in both Suntheanine and the same reference standards as were used in the assay of enzyme activity. While protein was identified in all three reference standards, no protein bands were confirmed for Suntheanine.

Since the electrophoresis revealed no protein content in Suntheanine and there was no enzyme activity, it is concluded that no glutaminase residues remain in Suntheanine.

b. Other Impurities

The spectrometric and HPLC analyses that were summarized above to demonstrate that Suntheanine is indeed theanine also provided evidence of the purity of the material by the absence of other peaks.

- With an LOD of between 1 ng and 1 pg of a substance, the mass spectrometry analysis of Suntheanine dissolved in glycerin had no peaks other than those of theanine and glycerol.
- Similarly, no peaks other than that of theanine were observed in the infrared spectrum of Suntheanine.
- In the NMR spectrometry, the spectra of Suntheanine and Theanine R (used as a reference) showed the same peaks, including the signal peak of the acetone used as a reference reagent, again suggesting that Suntheanine contains no unique impurities.
- In HPLC analyses using the AccQ-Tag methodology, in which five lots of Suntheanine were tested with an LOD for impurities of 0.15 ppm, the only peaks other than that of theanine that were observed were very small peaks that were also observed in all other chromatograms, including water, and which were therefore regarded as noise. This finding again indicates the absence of impurities in Suntheanine.

- Finally, reversed-phase HPLC analyses were conducted to determine the presence of glutamine or pyroglutamate, a substance that might be produced by the enzymatic treatment of L-glutamine in the production of Suntheanine. The LOD for these substances was 1.1 ppm; no peak corresponding to either substance was observed.

It is concluded that Suntheanine is extremely pure, with any impurities present at insignificant concentrations.

4. Food-Grade Specifications

Taiyo has developed food-grade specifications for Suntheanine. These specifications are intended to establish and maintain the food-grade status of the final product. The food-grade specifications established for Suntheanine are listed in Table 4.

Table 4. Food-Grade Specifications for Suntheanine

Parameter	Specification	Method
Appearance	White crystalline powder	Observation
Appearance in solution	Transparent and colorless in solution	Observation
Taste and odor	Slight sweet taste with no odor	Sensory test
Identification (TLC)	Single spot, Rf = 0.40–0.65	JSFA, 6th Ed.
Assay (dry basis)	98%–102% L-theanine	Japanese Pharmacopoeia, 13th Ed.
Loss on drying (105°C for 3 hours)	<0.5%	JSFA, 6th Ed.
Residue on ignition	<0.20%	JSFA, 6th Ed.
Optical rotation (5% solution) $(\alpha)^{20}_D$	+7.7° to +8.5°	JSFA, 6th Ed.
pH (1% solution)	5.0–6.0	JSFA, 6th Ed.
Chloride (Cl)	<0.021%	JSFA, 6th Ed.
Arsenic (as AS_2O_3)	<4.0 µg/g	JSFA, 6th Ed.
Heavy metals (as Pb)	<1.0 µg/g	JSFA, 6th Ed.
Lead	<1.0 µg/g	JSFA, 6th Ed.
Standard plate count	Less than 3000 cfu/g	JFHA, 1990
Total coliforms	Negative in 0.1 g	JHFA, 1990
Mold, yeast	Less than 100/g	JHFA, 1990

TLC – thin layer chromatography, Rf – retardation factor, JSFA – Japanese Standards for Food Additives, JHFA – Japanese Food Hygiene Association

5. Results of Analysis of Suntheanine

To demonstrate conformance with the food-grade specifications listed in Table 4, Taiyo analyzed 14 non-consecutive lots of Suntheanine. The results of these analyses are displayed in Table 5, shown below and on the following page.

All 14 lots were fully within established food-grade specifications with respect to all tested attributes, showing that the production process is in control and capable of consistently producing food-grade product.

Table 5. Results of Analysis of 14 Lots of Suntheanine

Attribute	Specification	Lot [redacted]						
Identification (Rf)	0.40–0.65	0.51	0.49	0.49	0.49	0.49	0.49	0.49
Assay titration	98–102%	100.8%	100.5%	100.8 %	100.8%	100.4%	100.6%	100.6%
Loss on drying	<0.5%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	0.2%	<0.1%
Residue on ignition	<0.20%	0.16%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Specific rotation	+7.7° to +8.5°	+8.1°	+8.1°	+8.2°	+8.2°	+8.3°	+8.3°	+8.0°
pH	5.0–6.0	5.7	5.6	5.5	5.7	5.6	5.6	5.6
Chloride	<0.021%	<0.02%	<0.02%	<0.02%	<0.02%	<0.02%	<0.02%	<0.02%
Arsenic	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g
Heavy metals (as Pb)	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g
Lead	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g
Plate count	<3000 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Coliforms	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Mold/Yeast	<100/g	<100/g	<100/g	<100/g	<100/g	<100/g	<100/g	<100/g

Table 5, continued. Results of Analysis of 14 Lots of Suntheanine

Attribute	Specification	Lot [redacted]						
Identification (Rf)	0.40–0.65	0.49	0.49	0.49	0.49	0.49	0.49	0.50
Assay titration	98–102%	100.6%	101.3%	101.1%	101.4%	101.3%	101.1%	100.8%
Loss on drying	<0.5%	<0.1%	<0.1%	<0.1%	0.2%	0.2%	<0.1%	0.1%
Residue on ignition	<0.20%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%	<0.1%
Specific rotation	+7.7° to +8.5°	+8.2°	+8.2°	+8.2°	+8.2°	+8.1°	+8.3°	+8.3°
pH	5.0–6.0	5.5	5.6	5.6	5.5	5.5	5.6	5.6
Chloride	<0.021%	<0.02%	<0.02%	<0.02%	<0.02%	<0.02%	<0.02%	<0.02%
Arsenic	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g	<4.0 µg/g
Heavy metals (as Pb)	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g
Lead	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g	<1.0 µg/g
Plate count	<3000 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Coliforms	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Mold/Yeast	<100/g	<100/g	<100/g	<100/g	<100/g	<100/g	<100/g	<100/g

H. Product Stability

1. Stability of Suntheanine during Sterilizing Conditions

A 0.2% solution of Suntheanine (pH = 6.0) was sterilized at 121°C for 15 minutes. The theanine concentration was analyzed by HPLC. As shown in Table 6, the heat treatment had negligible effect on the theanine content of Suntheanine.

Table 6. Stability of Suntheanine at 121°C

Sterilizing Conditions	Amount of Theanine (%)	
121°C, 15 minutes	Before Sterilization	After Sterilization
	100	99

2. Stability of Suntheanine in Various pH Solutions

The stability of Suntheanine under neutral conditions as well as under various levels of acidity was tested over a 4-month storage period. Three 0.2% solutions of Suntheanine were

prepared and adjusted to pH 3.0 by citrate buffer and pH 6.5 by sodium carbohydrate buffer. The solutions were placed in polypropylene bags and sealed, then kept at room temperature (approximately 25°C) under dark conditions for 4 months. The theanine concentration was analyzed by HPLC immediately and again at 4 months. The results, shown in Table 7, showed that only slight degradation occurred in the theanine content of the product. The degradation products were pyroglutamic acid and ethylamine.

Table 7. Stability of Suntheanine at Neutral and Low pH

pH of Solution	Amount of Theanine (%)	
	Initial	After 4 Months
3.0	100	88.0
6.5	100	99.1

3. Storage Stability of Suntheanine

Samples of Suntheanine produced in 1996 (lot 960610) and in 1998 (lot 805251) were packaged in tightly closed containers and stored in the dark at room temperature (25°C) for 2 years. The theanine concentration was analyzed initially and at 1 and 2 years by HPLC, using L-norleucine as an internal standard. As shown in Table 8, no consistent changes were seen in the L-theanine peak area or peak height or in the ratio of the L-theanine area to the L-norleucine area. This study confirms a shelf life of at least 18 months.

Table 8. Storage Stability of Suntheanine

Measure / Lot & Year	Δ L-Theanine Peak Area	Δ L-Theanine Peak Height	Δ (L-Theanine Area/ L-Norleucine Area)
Lot 960610 (1996)			
After 1 year	+ 0.1%	- 0.2%	- 1.1%
After 2 years	- 1.2%	+ 2.9%	- 2.4%
Lot 805251 (1998)			
After 1 year	- 0.3%	- 1.0%	+ 0.4%
After 2 years	+ 0.5%	0	+1.0%

III. INTENDED TECHNICAL EFFECT

The intended technical effect of the addition of Suntheanine to foods is to increase the dietary intake of the amino acid L-theanine.

A number of beneficial effects have been suggested for L-theanine; some of these effects are well supported by research and others less so. Perhaps the most studied effect of L-theanine is its ability to produce relaxation and to act as an antagonist to caffeine. Kobayashi et al. (1998) divided 50 females age 18–22 into a high-anxiety group and a low-anxiety group based on scores on the Manifest Anxiety Scale. Four high- and four low-anxiety subjects were chosen and given either 50 mg or 200 mg theanine in water once a week. Each subject's electroencephalogram (EEG) was taken for 60 minutes after each administration. Effects were observed beginning about 30–40 minutes after intake, taking the form of increased production of alpha waves (reported to be characteristic of relaxation), but not theta waves (reported to be indicators of drowsiness). The experimenters suggest that this effect may be partly masked by caffeine when theanine is obtained by drinking tea.

Song et al. (2002) performed a randomized placebo-controlled double-blind crossover study with 20 subjects age 30–55 years suffering from persistent fatigue. The subjects were classified as high or low in anxiety. Each subject's frontal and occipital EEG was measured over 1 hour immediately after the administration of a placebo or 200 mg L-theanine every day for 7 days. The subjects were also evaluated on the Fatigue Severity Scale. Both endpoints showed significant relaxant effects of theanine; the fatigue score was significantly ($p<0.05$) decreased in the test group but not in the placebo group, and there were significant ($p<0.05$) differences in the α to β power value, chosen as a surrogate marker of mental relaxation, in the high-anxiety group but not in the low-anxiety group. In a follow-up study with younger males (age 18–30), Song et al. (2003) performed a second randomized placebo-controlled double-blind crossover study with 20 subjects who were given theanine or placebo. The subjects were again classified as high or low in anxiety. Significant ($p<0.05$) differences were observed in the occipital α signals in those with high anxiety, but not in those with low anxiety. Song et al. (2003) concluded that the results of both studies indicate that theanine promotes the release of α waves related to mental relaxation and concentration.

M. Weiss and his colleagues at the Department of Sports and Health, University of Paderborn, conducted a number of studies of the effects of L-theanine on the recovery of individuals from the stress of heavy exercise. In an unpublished study (Weiss et al. undated), 14 healthy male athletes exercised on a bicycle ergometer and then consumed a drink with 0, 50, or 200 mg L-theanine in a randomized double-blind crossover design. Theanine had no effect on hormones important for regeneration, but lowered prolactin (which is regulated by dopamine and serotonin). There was no significant change in brain function, but lowered β waves, reported to be an indication of relaxation, were observed.

In a similar randomized double-blind crossover study, Weiss et al. (2001a) gave 14 healthy athletes drinks with 0, 50, or 200 mg theanine after exhausting exercise on a bicycle-ergometer. Electrosympathicography (ESG) was used to measure skin resistance at 0, 15, 30, 45, 60, 75, and 135 minutes post-administration. L-theanine had no effect on peripheral sympathetic

electrodermal activity during regeneration. In another phase of this same study, but reported separately (Weiss et al. 2001b), plasma levels of catecholamines, cortisole, prolactin, and serotonin were measured 0, 30, 45, 60, 120 minutes after the drink. No effects of theanine on hormonal levels were observed. Finally, in a third phase of the study (Weiss et al. 2001c), EEG-mapping was used to assess the effect of ingestion of L-theanine; the experimenters reported that "L-theanine seemed to accelerate the normalization of EEG spectral power in high frequency waves," supporting physiological relaxation after severe exercise.

In another unpublished study, Weiss and Geiss (undated) sought to determine if theanine administration had any adverse effects on cognitive function, such as poorer reaction time, concentration, alertness, or attention. In a randomized double-blind placebo-controlled crossover study, 20 male students age 19–32 ingested either placebo or 200 mg L-theanine at breakfast. No significant differences were observed in heart rate or blood pressure, blood glucose, red blood cells, hemoglobin, hematocrit, uric acid, urea, or γ-glutathione. No differences were observed in reaction time or accuracy of response, concentration, or perceptual speed on a tachistoscopic test.

Several investigators have speculated that improved relaxation might manifest itself in better performance in tests of learning, memory, or physical control. Juneja et al. (1999) report a study in which 180 mg theanine/day was administered to weanling male Wistar rats for 4 months. As compared with controls, the theanine-treated rats did better on an operant test that required them to push a lever to get food when a light is on, which is regarded as a test of both learning ability and memory. Animals receiving theanine also outperformed controls on both passive avoidance and active avoidance tests, both presented as tests of memory. A similar experiment was conducted by Terashima et al. (1999a), in which 3-week-old male Wistar rats were given *ad libitum* access to water with 10 mg theanine/ml. After 3 months, the rats were given a learning test and the active and passive avoidance tests of memory. Little effect was noted on learning ability, but there was a larger effect on the rats' performance on tests of memory.

Kim et al. (2001) conducted a unique study in which they used golfers instead of rats. In a randomized double-blind crossover study, 23 healthy male golfers received 300 mg Suntheanine + 20 mg L-carnitine + 100 mg Dongchunghacho or a placebo for 2 days. Those receiving the theanine-containing beverage showed significant increases in α waves of the frontal and occipital regions beginning 30 minutes after administration. Of most interest to golfers, there were significant improvements in putting success rate and in driving accuracy.

As noted above, Kobayashi et al. (1998) speculated that caffeine and theanine are antagonistic. Kakuda et al. (2000) intravenously administered caffeine to rats at a dose of 5 μmol/kg bw (0.970 mg/kg bw), a dose reportedly about equal to the concentration of caffeine in a cup of coffee, and observed excitatory effects in the EEGs lasting for at least 180 minutes. When they co-administered 5 μmol theanine/kg bw (0.871 mg/kg bw), the stimulation was inhibited, and at a theanine dose of 50 μmol/kg bw (8.710 mg/kg bw) the caffeine stimulation was almost completely suppressed. Paradoxically, however, theanine administered alone at a low dose of 2 μmol/kg bw (0.348 mg/kg) had a modest stimulatory effect. Since Yokogoshi and Terashima (2000) found that administration of theanine results in a dose-dependent increase of

dopamine in the brain, especially in the striatum, hypothalamus, and hippocampus. Kakuda et al. (2000) suggested that their findings might be due to this increased release of dopamine.

On the other hand, in an *in vitro* study using slices of rats' cerebral cortex, Kimura and Murata (1980) found that theanine does not directly affect or antagonize caffeine in the formation of adenosine 3',5'-monophosphate, but that theanine does inhibit its formation mediated by norepinephrine and histamine. In a later study, Kimura and Murata (1986) found that theanine decreases the levels of norepinephrine in the brains of rats, apparently by releasing it rather than by inhibiting its synthesis. They also found that theanine does not directly affect levels of serotonin, but inhibits the increase that is caused by caffeine administration. This conclusion was supported by the work of Yokogoshi et al. (1998b) in three experiments with young adult male Wistar rats weighing about 100 g. In Experiment 1, rats were gavaged with 2000 mg/kg bw theanine or saline and decapitated after 2 hours. In Experiment 2, rats were gavaged with 2000, 4000, or 8000 mg/kg bw theanine or saline, and brain 5-hydroxyindoles were measured after 2 hours. In Experiment 3, rats also received pargyline along with the theanine or saline, and serum tryptophan, brain tryptophan, serotonin, and 5-hydroxyindole acetic acid were measured. Theanine induced reduction of brain serotonin and 5-hydroxyindole concentrations, but increased brain tryptophan. Yokogoshi et al. (1998b) concluded that theanine reduced serotonin synthesis and also increased serotonin degradation.

Nozawa et al. (1995) studied the effects of theanine on brain function *in vitro* using cultured cortical neurons. The addition of theanine was found to increase the Ca^{++} in the neurons. Nozawa et al. (1995) concluded that their findings suggest that theanine is a glutamate analog that is stimulatory at low concentration, but sedative at high concentration, suppressing excitation produced by caffeine. Kimura et al. (1975), in research with mice, found that theanine inhibits the excitory effect of caffeine, but does not affect the spontaneous activity of mice when caffeine is not being administered. Kimura and Murata (1971a) reported that the inhibitory action of theanine against caffeine is specific, and that theanine does not inhibit the convulsive action of pentetrazole, picrotoxin, strychnine, pipradrol, or bemegride. Theanine also does not potentiate the effect of the hypnotic hexobarbital sodium.

Sagesaka et al. (1991) compared the effect of orally administered theanine to theanine administered intraperitoneally or subcutaneously with respect to its antagonistic reaction to caffeine in male ICR-JCL mice. Administration of 4 mg/kg bw caffeine increased the level of spontaneous motor activity. This was counteracted by 1740 mg/kg bw theanine, but not by 174 mg/kg bw.

Related to theanine's effects discussed above is its role in neuroprotection, mediated at least in part by its affinity to bind to glutamate receptors and reduce the level of glutamate, an excitatory neurotransmitter that can cause cellular damage at high concentrations. The earliest indication of this effect was a study in crayfish, which found that theanine competes with glutamate for the glutamate receptors at the neuromuscular junctions (Shinozaki and Ishida 1978). This was followed by an *in vitro* study with cultured rat cerebral cortical neurons in which Nozawa et al. (1998) demonstrated that theanine inhibits glutamate-induced neurotoxicity and may thus reduce ischemia. Zhang et al. (2001) studied *in vitro* and *ex vivo* effects of theanine on invasion of a rat ascites hepatoma cell line of AH109A. They observed a dose-dependent

inhibition of the invasion of AH109A cells across rat mesentery-derived mesothelial-cell monolayers, an effect believed to be mediated by the glutamate receptor of AH109A.

Kakuda et al. (2002) also used cerebral cortexes of Wistar rats in another *in vitro* study, in which they found that theanine binds to all types of glutamate receptors, but its binding capacity is less than that of L-glutamic acid by a factor of 80- to 30000-fold. They suggested that this relatively poor binding capacity implies that competition for binding sites produces only a mild effect and indicates that there may be other mechanisms at work. In another study published later that same year, Kakuda (2002) showed that the death of hippocampal CA1 pyramidal neurons caused by transient forebrain ischemia in the gerbil was inhibited with the ventricular preadministration of theanine. Theanine has a higher binding capacity for the AMPA/kainate receptors than for NMDA receptors, and this same treatment also prevented neuronal death in the hippocampal CA3 region by kainic acid. Kakuda (2002) suggested that the mechanism of the neuroprotective effect of theanine is related not only to the glutamate receptor but also to the glutamate transporter.

This latter study confirmed earlier *in vivo* work by Nozawa et al. (2001) in which a catheter was inserted in rats' lateral ventricles and theanine or saline was administered. After 5 days, kainic acid was administered intraperitoneally. After 7 days, tissue samples were prepared and kainic-acid-induced neuronal death in the CA3 and CA4 regions of the hippocampus was observed. The severity of the kainic-acid effect was reduced in the rats treated with theanine.

Nagasawa et al. (2004) studied theanine's ability to protect neurons from the excitotoxicity of glutamate *in vitro* using cultured rat cortical neurons and focusing on group I metabotropic glutamate receptors. Their findings indicate that these group I receptors may be involved in the neuroprotective effect of theanine by increasing the expression levels of phospholipase C-β1 and $-\gamma$1. In an *in vivo* study in male ddY mice, Egashira et al. (2004) injected theanine 3 hours after occlusion of the middle cerebral artery (as a model of ischemic brain damage). Theanine significantly decreased the size of the resulting cerebral infarcts, but did not affect cerebral blood flow or brain temperature.

In an investigation of the effects of theanine on neurotransmitter release in the rat brain striatum, Yamada et al. (2005) performed *in vivo* brain microdialysis and compared inhibition of glutamate transporters by theanine with that resulting from a known blocker, L-trans-Pyrrolidine-2,4-dicarboxylic acid. Both substances caused dopamine release from dopaminergic neurons, but L-trans-2,4-PDC perfusion increased glutamic and aspartic acid while theanine prevented aspartic acid release and increased glycine release, suggesting that the two substances operate via different mechanisms. While L-trans-2,4-PDC causes excitatory neurotransmission, theanine may inhibit excitatory neurotransmission and cause inhibitory neurotransmission via glycine receptors.

It has also been demonstrated that theanine can serve as a potentiator for antitumor effects of doxorubicin, cisplatin, and irinotecan, primarily by inhibiting the efflux of the drug from the tumor cells (Sugiyama and Sadsuka 1998, Sugiyama and Sadzuka 1999, Sadzuka et al. 2002a and 2002b). Sugiyama and Sadzuka (2003) showed that, in M5076 ovarian sarcoma-

bearing mice, theanine increases the concentration of doxorubicin in the tumor cells, but not in normal tissues. Theanine inhibited the efflux of doxorubicin from tumor cells and inhibited the glutamate uptake by M5076 cells by inhibiting glutamate transporters. Sugiyama et al. (2000) found that theanine enhanced the antitumor effects of doxorubicin by inhibiting the glutamate transport, thus reducing glutamate uptake. Sugiyama et al. (1999) found that theanine enhances the activity of idarubicin against acute myelocytic leukemia while reducing the adverse side effects of leukopenia and suppression of bone marrow cells. Sugiyama et al. (2003) also found that theanine reduces adverse reactions to doxorubicin, such as induction of the lipid peroxide level and reduction of glutathione peroxidase activity in normal tissues. This finding was confirmed by Sugiyama and Sadzuka (2004), who also found that theanine reduces the glutamate concentration in the tumors of M5076 tumor-bearing mice, but increases it in the heart and liver. The experimenters suggested that theanine may be converted to glutathione, which plays an important role in detoxification and repair of cellular injury. Importantly, Sadzuka et al. (2006) showed that the decrease of doxorubicin's adverse reactions by theanine was not connected with activity of cytochrome P450 enzymes and concluded that "theanine has no effect on the metabolism of other medicines and is safe as a food (tea) or supplement."

Another beneficial effect that has been suggested for theanine, less well studied than the foregoing, is alteration of lipid metabolism and consequent aid in controlling obesity. In a prospective label-blinded clinical trial, Krieger et al. (2001) gave 12 overweight adults the weight-loss product Norexin™ with 15 mg ephedrine, 150 mg caffeine, and 10 mg theanine two times a day for 14 days. This treatment induced weight and fat loss with no change in caloric intake and no apparent sympathomimetic effects—heart rate variability, blood pressure, blood sugar, EKG, temperature, sleep habits, or perceived stress level. Krieger et al. (2001) did not attempt to determine the specific effects of the various components of the treatment. In a later *in vitro* study, Kim et al. (2003) treated differentiated 3T3-L1 cells with an extract of green tea or with the extract's main active compounds, catechins, caffeine, and theanine. Glycerol release was increased in the cells treated with the green-tea extract; caffeine and theanine also showed lipolytic activity. Kim et al. (2003) suggest that the stimulation of lipolysis by theanine and caffeine, along with inhibition of adipogenesis by its catechins, give green tea an anti-obesity effect. In an *in vivo* study in which female ICR mice were fed diets with 2% green-tea powder, 0.3% catechins, 0.05% caffeine, or 0.03% theanine, Zheng et al. (2004) determined that both caffeine and theanine suppress body-weight increase and fat accumulation and that they are synergistic in anti-obesity activity.

It has been suggested that theanine might have a protective effect with regard to cardiovascular disease, but the only research to date has not been supportive of this suggestion. Yokozawa et al. (1995) investigated the ability of the components of tea to inhibit proliferation of smooth muscle cells, which may help protect against atherosclerosis. He found that, while the catechins appeared to have such an inhibitory effect, theanine had none. Similarly, Yokozawa and Dong (1997) studied the effect of tea components on the peroxidation of low-density lipoproteins and observed dose-dependent inhibition of such peroxidation by tea polyphenols and, only weakly, theanine.

Although there has been little research on the immunomodulatory effects of orally ingested theanine, there is a reasonable theoretical basis for expecting such effects. One possible

mechanism for an immunomodulatory effect of theanine was explored by Bukowski et al. (1999). One class of T cells, γδ T cells, responds to alkylamines secreted by bacteria. These alkylamines, which are also found in some edible plants, include ethylamine. Since ethylamine is a metabolite of theanine, stimulation of γδ T cells is likely to occur in response to theanine. Bukowski et al. (1999) demonstrated this effect *in vitro* by hydrolyzing green and black tea extracts to liberate ethylamine from theanine; this hydrolysate induced a 3- to 5-fold expansion of γδ T cells. Similarly, while intact theanine did not cause γδ T cell expansion, theanine that was acid hydrolyzed caused a 15-fold expansion of γδ T cells. To verify that ethylamine was released by acid hydrolysis of tea extracts and of pure theanine, a quantitative headspace GC-mass spectrometry analysis detected ethylamine in the hydrolysates but not in the untreated tea extract or theanine. Kamath et al. (2003) carried this work forward with an *in vivo* study in which 11 healthy non-tea-drinking individuals drank 5–6 cups/day of tea for either 2 or 4 weeks. Blood was drawn weekly. Theanine, a precursor of ethylamine, primed peripheral blood γδ T cells to mediate a memory response on re-exposure to ethylamine and to secrete IFN-γ in response to bacteria. Thus, theanine results in an enhanced innate immune response to bacteria that are γδ T cell-dependent.

Three effects of theanine, each addressed in only one published study, may be related to theanine's role as a modulator of neurotransmitters and its relaxant effect. Juneja et al. (1999), reports a study in which various amounts of theanine were injected intraperitoneally into spontaneously hypertensive rats; their blood pressure was measured before the injection and 60 minutes later. Glutamine was administered as a control. Theanine produced a dose-dependent reduction in blood pressure, but glutamine did not.

Shiraga et al. (2003) produced elevated intraocular pressure in rats by cautery of three episcleral vessels and measured secondary degeneration of the retinal ganglion cells with or without ingestion of 1 mM/day theanine for 5 months. Retinal ganglion cell loss in the eyes of rats treated with theanine was significantly less than in untreated rats, indicating that theanine may have neuroprotective value in a rat model of chronic glaucoma.

In a study of the role of theanine in mitigating the effects of premenstrual syndrome (PMS) (Ueda et al. 2001), 20 women age 22–49 (mean 30.0 years) were administered 2 tablets with 50 mg Suntheanine or placebo 2 times a day (200 mg theanine/day) through three menstruation cycles. Cycle 1 was used as a baseline, and a crossover was used between cycles 2 and 3. Subjects completed a Menstrual Distress Questionnaire with 47 questions addressing the physical symptoms of pain, concentration, behavior change, autonomic reactions, water retention, and negative affect, and the mental symptoms of arousal and control. Theanine significantly reduced scores on both physical and mental symptoms of PMS.

IV. Intended Use and Consumer Exposure

A. Existing Sources and Intake of L-Theanine

1. Sources of L-Theanine

Theanine is an amino acid that is found at detectible levels in tea plants (*Camellia sinensis*) and in one inedible species of mushroom, *Xerocomus badius* (Neumann and Montag 1982, Feldheim et al. 1986, Ekborg-Ott et al. 1997, Chen et al. 2003, Kato et al. 2003). It is biosynthesized in the root of the tea plant by the enzymatic action of theanine synthetase on glutamic acid and ethylamine, which in turn is produced by the enzymatic decarboxylation of alanine (Feldheim et al. 1986, Tsushida 1987, Ekborg-Ott et al. 1997). Theanine is then translocated to the shoots, shoot tips, and leaves, where it serves as the major source of nitrogen (Tsushida 1987, Ekborg-Ott et al. 1997), although isotopic studies have found that N-ethyl-^{14}C of theanine was incorporated significantly into polyphenols (Feldheim et al. 1986). Theanine exists only in free form—i.e., it is not incorporated into proteins (Graham 1992, Ekborg-Ott et al. 1997)—and it constitutes half or more of the total free amino acids and about 1–2.5% of the total dry weight of the tea leaf.

There are three major types of tea, green (unfermented), half-green or Oolong (semi-fermented), and black (fermented); "fermentation" really refers to oxidation of the polyphenolic compounds belonging to the catechin groups rather than to a process of digestion by microorganisms (Graham 1992, Ekborg-Ott et al. 1997). The theanine content of black tea has been believed to be less than in the other types due to degradation into glutamic acid and ethylamine during oxidation (Ekborg-Ott et al. 1997). Results of recent analyses of the theanine content of different types of tea, however, appear to contradict this assumption.

Neumann and Montag (1982) used HPLC to analyze theanine concentrations in 17 varieties of black, half-green, and green teas. Their findings are summarized in Table 9, which shows that theanine ranged from 0.19% to 1.40% of the dry weight of the teas they tested and constituted from 33.0% to 60.3% of the free amino acids. The overall average theanine concentration in Neumann and Montag's (1982) sample was 0.70%; black teas had the highest average theanine content (0.86%) and half-green teas had the lowest (0.45%). Theanine constituted an average of 48.3% of the free amino acids, ranging from 42.5% in green tea to 51.0% in black tea. Neumann and Montag (1982) used what they referred to as an "automatic amino acid analyzer," and it is possible that this technique did not provide adequate separation, because the theanine concentrations reported in this study are lower than those found in most of the later research.

Table 9. Theanine Concentrations in Tea (Neumann and Montag 1982)

Tea Name	Tea Type	Theanine (g/100g tea)	Theanine (g/100g free amino acids)
Keemun	black	1.22	50.8
Assam	black	1.40	60.1
Darjeeling	black	0.54	46.7
Ceylon	black	0.62	52.1
Ceylon decaffeinated	black	0.73	53.8
Java	black	1.18	57.2
Turkey	black	0.89	51.2
USSR	black	0.32	35.6
Kenya	black	0.86	51.8
Oolong	half-green	0.34	46.4
Tungting	half-green	0.36	47.0
Pouchung	half-green	0.66	55.6
Gunpowder	green	0.25	38.0
Taiwan green	green	1.34	60.3
Chun Mee	green	0.19	33.0
Loong Tseng	green	0.79	46.7
Sencha Makoto	green	0.28	34.7

Source: Neumann and Montag 1982

Using a different technique, thin-layer chromatography and densiometry, Feldheim et al. (1986) analyzed 20 samples of black tea for their theanine content. This methodology resulted in even lower estimates of theanine concentration: an average of 0.75% compared with the 0.86% found in black teas by Neumann and Montag (1982). The results for all 20 tea samples are shown in Table 10.

Table 10. Theanine Concentrations in Tea (Feldheim et al. 1986)

Tea Name	Theanine (g/100g tea)
Darjeeling Gastelton	0.25
Darjeeling Seeyok	0.74
Darjeeling Lingia	0.38
Ceylon Neluwa	0.45
Ceylon Highlands	0.53
Java	1.04
Ceylon Dyraaba	0.43
Assam Kamu	1.51
Assam Doyang	0.95
Zimbabwe Dust	1.59
S. Indian Nov	0.82
Assam OF	1.03
Ceylong D	0.33
Africa Kenya D	1.11
Vietnam	0.54
Bangladesh	0.68
Mozambique	0.42
Brazil Dust	0.86
China Dust	0.61
S. Indian Nov	0.69

Source: Feldheim et al. 1986

Another approach to measuring the concentration of theanine in tea was adopted by Kato et al. (2003), who used microchip electrophoresis to measure the free amino acids in Japanese green tea. They reported an average theanine concentration of 1.475%, more than double the 0.57% concentration found in green teas by Neumann and Montag (1982). Kato et al. (2003) also found arginine at 0.408% and glutamine at 0.217% and no other free amino acids at detectible levels.

The theanine content of green tea found by Kato et al. (2003) is similar to that reported earlier in an HPLC-based study by Ekborg-Ott et al. in 1997, which found an average of 1.42% theanine in two varieties of green tea.

Using capillary electrophoresis, Chen et al. (2003) analyzed the composition of theanine (along with caffeine and polyphenols) in various parts of fresh green tea. Specifically, they analyzed tea leaves by age counting from the end of the shoot tip back toward the main stalk,

with the bud at the end of the tip being the youngest and the 10th leaf the oldest. As shown by the results given in Table 11, there does not appear to be a linear pattern of increasing or decreasing theanine content with age of leaf. What is notable, however, is that the average theanine content of these fresh green tea leaves was 3.755%, more than twice the level found in other studies.

Table 11. Theanine Concentrations in Fresh Tea Leaves (Chen et al. 2003)

Leaf	Theanine (g/100 g tea)
bud (youngest)	4.73
1st leaf	4.23
2nd leaf	2.64
3rd leaf	1.89
4th leaf	1.42
5th leaf	2.80
6th leaf	4.94
7th leaf	3.82
8th leaf	5.84
9th leaf	3.53
10th leaf (oldest)	5.46

Source: Chen et al. 2003

Chen et al. (2003) also used capillary electrophoresis to measure the theanine content of six varieties of Taiwanese and Chinese Oolong teas (Table 12). They again found higher levels that have been reported in other studies, with averages of 6.09% in the Taiwanese teas and 6.42% in the Chinese teas, an overall average of 6.26%.

Table 12. Theanine Concentrations in Oolong Teas (Chen et al. 2003)

Type of Tea	Theanine (g/100 g tea)
Taiwan 1	5.28
Taiwan 2	6.01
Taiwan 3	6.99
China 1	2.04
China 2	9.20
China 3	8.01

Source: Chen et al. 2003

Like most amino acids, theanine is chiral, containing a stereogenic center and existing in two enantiomeric forms, D-theanine and L-theanine (Ekborg-Ott et al. 1997, Desai and

Armstrong 2004). Due to the chiral nature of the substance, the pharmacologic and other effects of D- and L-theanine may differ (Desai and Armstrong 2004); for example, many D- amino acids have a sweet taste, while the L- form is bitter (Ekborg-Ott et al. 1997). The latter researchers used HPLC to study the total theanine concentrations and enantiomeric composition of theanine in a variety of black, half-green, and green teas (Ekborg-Ott et al. 1997). As shown in Table 13, the differences between types of tea in total theanine content were not striking.

Table 13. Theanine Concentrations and Enantiomeric Compositions in Different Types of Tea (Ekborg-Ott et al. 1997)

Tea Name	Tea Type	Theanine (g/100 g tea)	% D-/DL-Theanine
African Flower	black	1.30	0.54
Assam	black	1.05	0.49
Ceylon Broken	black	1.32	2.30
Ceylon Pekoe	black	2.20	0.34
Cherry Blend	black	2.04	0.21
Darjeeling	black	1.45	0.45
Earl Grey	black	1.07	0.42
Georgian	black	1.16	0.46
Keemun	black	1.12	0.65
Lapsang Souchong	black	0.82	1.04
Lemon Blend	black	1.26	2.70
Rosen	black	1.03	2.46
Yunnan	black	2.38	1.79
Formosa Oolong	half-green	0.60	12.70
Jasmine	half-green	1.72	0.45
Gunpowder	green	1.78	2.20
Sencha	green	1.05	2.20

Source: Ekborg-Ott et al. 1997

The average theanine content of black teas was 1.40%, as compared to 1.42% in green teas and 1.16% in half-green teas. Overall, D-theanine constituted only 1.85% of the total theanine content in the tea leaves; thus, over 98% of the theanine was the L- enantiomer.

In conclusion, it is apparent that the theanine content of tea leaves is highly variable, and does not appear to be consistent across different types of tea. Values ranging from about 0.7% to greater than 6% have been reported in the literature, with most values clustered around about 1.0–2.5%. L-theanine predominates in all varieties of tea, and overall appears to constitute in excess of 98% of the total theanine present.

It might be noted that this predominance of L-theanine is found in naturally occurring theanine produced by biosynthesis. In chemically synthesized theanine, the mixture of enantiomers may be essentially racemic, as was found in analyses of theanine products from six manufacturers (Desai and Armstrong 2004) using HPLC and atmospheric pressure chemical ionization mass spectrometry (APCI-MS). The only theanine product that did not produce significant D- peaks was Suntheanine; all other products showed approximately equal abundance of D- and L-theanine.

Since theanine is readily soluble in water (Ekborg-Ott et al. 1997), it is likely that most of the theanine in the tea leaves dissolves in the water when tea beverages are prepared, and this is the only source of theanine in the human diet.

2. Tea Beverage Consumption

Tea is grown in more than 30 countries, the top producers being India, China, Sri Lanka, Kenya, Indonesia, and Turkey (Graham 1992, Desai and Armstrong 2004). Over 3 million tons of tea are produced annually (FAO 2005). All varieties are now regarded as a single species, *Camellia sinensis*, and—other than water—tea is the most widely consumed beverage in the world (Graham 1992). The UK Tea Council estimates that approximately 40% of that nation's fluid intake comes from tea (UK Tea Council). Blumberg (2003) estimated that worldwide per capita tea consumption was about 120 ml/day, approximately 77% black tea, 21% green tea, and 2% oolong tea.

Exponent Inc. estimated consumption of both leaf and ready-to-drink (RTD) tea beverages in the United States based on data collected in the NHANES in 1999–2002 available on the NHANES website (DHHS 2005). This survey was conducted by the National Centers for Health Statistics, Centers for Disease Control and Prevention, with non-institutionalized individuals in the United States. Twenty-four-hour diet recall data were collected through in-person interviews from a nationally representative sample of individuals of all ages.

Daily intakes of tea both on a per-person and on a body-weight basis were calculated for the total population as well as for males and females age 13 and older. The mean daily intake and upper percentiles of intake are shown in Tables 14 and 15.

Table 14. Tea Beverage Consumption in the U.S. by Person

Population Group	% Users	Daily Intake of Users (ml)			
		Mean	90th Percent	95th Percent	99th Percent
Total Population	18.7	763	1650	1907	3337
All 13+	21.2	795	1654	1929	3558
Females 13+	22.7	712	1482	1777	3108
Males 13+	19.7	896	1726	2376	3817

Source: DHHS 2005

Table 15. Tea Beverage Consumption in the U.S. by Kg Body Weight

Population Group	% Users	Daily Intake of Users (ml/kg bw)			
		Mean	90th Percent	95th Percent	99th Percent
Total Population	18.7	10.6	20.9	26.4	47.7
All 13+	21.2	10.2	20.2	25.6	47.0
Females 13+	22.7	9.8	19.7	24.9	40.5
Males 13+	19.7	10.7	20.6	27.6	47.9

Source: DHHS 2005

Just under 20% of the total United States population and slightly over 20% of the population age 13 and older reported drinking tea on the survey day. This includes all varieties of tea beverages, including those made from leaf tea, powdered instant tea, hot tea, iced tea, regular tea, presweetened tea, and decaffeinated tea. The mean reported daily tea consumption among users was 763 ml, or 10.6 ml/kg bw. The 95th percentile reported consumption of tea in the United States population was 1907 ml/day or 26.4 ml/kg bw/day.

3. Current Intake of L-Theanine

As was noted earlier, tea leaves contain—on average—about 1–2.5% theanine, nearly all of it the L- enantiomer, and nearly all of it likely to dissolve in the water in which the tea is brewed or dissolved. Researchers at the Linus Pauling Institute concluded that:

> "Typically, tea is made at a concentration of about 2%; that is, 2 grams (about 1/10 oz) of tea leaves are brewed in 100 milliliters (about 3 1/2 oz) of water—boiling for black tea (followed by 3–5 minutes of brewing), below boiling for green tea." (Dashwood 2003)

Based on this approximation, 2 g tea leaves ↔ 100 ml beverage, the tea consumption data shown in Tables 11 and 12 can be used to estimate the amount of tea required to brew the listed amounts of beverage. This information in turn can be used to estimate current intake of L-theanine from consumption of tea based on the average theanine concentration of 1.0–2.5% in tea leaves. Since there is no consumption of the single inedible variety of mushroom (*Xerocomus badius*) also known to contain theanine, these figures represent total current United States intakes of L-theanine.

Estimated tea consumption levels for the United States population are shown in Table 16. The mean daily consumption of 763 ml of tea beverage implies the use of 15.26 g of tea leaves, which provide between 152.6 and 381.5 mg of L-theanine. At the 99th percentile of tea

consumption, daily intake of L-theanine is estimated to be between 667.4 and 1668.6 mg (9.54–23.85 mg/kg bw).

Table 16. Tea Beverage Consumption in the U.S., Estimated Use of Tea Leaves, and Estimated Intake of L-Theanine

Consumption Percentile	Tea Beverage (ml)	Tea Leaves (g)	L-theanine (mg)	
			@ 1.0% Content	@ 2.5% Content
Per person				
Mean	763	15.26	152.6	381.5
90th percentile	1650	33.00	330.0	825.0
95th percentile	1907	38.14	381.4	953.5
99th percentile	3337	66.74	667.4	1668.5
Per kg bw				
Mean	10.6	0.21	2.12	5.30
90th percentile	20.9	0.42	4.18	10.45
95th percentile	26.4	0.53	5.28	13.20
99th percentile	47.7	0.95	9.54	23.85

B. Intended Uses and Estimated Intakes of Suntheanine

1. Intended Uses

Suntheanine is intended to be added to foods in the categories shown in Table 17 at a level not to exceed 250 mg/serving.

Table 17. Intended Food Uses of Suntheanine

Food Category	Serving Size (RACC)	Intended Addition Level (mg/g)
Fruit juices	240 ml	1.0
Sports beverages	240 ml	1.0
Non-herbal teas	240 ml	1.0
Bottled waters	240 ml	1.0
Chocolate power bars and chews	50 g	5.0
Breath mints	2 g	125.0
Hard candies	15 g	16.7
Chewing gum	3 g	83.3

2. Estimated Daily Intake of Suntheanine

Exponent Inc. calculated the daily intake of Suntheanine estimated to result from its intended addition to food. These estimates were based on data collected in the United States Department of Agriculture's 1994–96 Continuing Survey of Food Intakes by Individuals (CSFII) and its Supplemental Children's Survey (CSFII 1998), as provided on CD-ROM (USDA 2000). The CSFII 1994–96 was conducted between January, 1994, and January, 1997, with non-institutionalized individuals in the United States. Twenty-four-hour diet recall data were collected through two in-person interviews conducted between 3 and 10 days apart from a nationally representative sample of individuals of all ages. The CSFII 1998 was designed as a supplement to CSFII 1994–96 to increase the sample size for children from birth through age 9 years. This survey employed the same methodology as CSFII 1994–96. In the merged surveys (designated CSFII 1994–96, 1998), 21662 individuals provided dietary intake data on the first survey day, and 20607 provided data on the second day. The CSFII was selected, rather than the more recent NHANES 1999–2002, because the availability of 2-day data allows better estimation of the usual intake of Suntheanine potentially resulting from its intended use.

EDIs are calculated by multiplying the reported consumption of each food in categories in which Suntheanine addition is intended by the maximum intended addition level as shown in Table 17. The total EDI is calculated by adding, at the level of the individual respondent, the intakes of Suntheanine from each use in foods consumed by the individual. The results of these calculations are shown in Table 18.

Table 18. Estimated Daily Intake of Suntheanine from Its Intended Use

Food Category	% Users	Daily Intake of Suntheanine (mg)			
		Per User (mg/day)		Per Kg Body Weight (mg/kg bw/day)	
		Mean	90th Percentile	Mean	90th Percentile
Bottled water	<0.1	121	242	8.1	16.2
Chocolate bars & chews	4.0	181	304	3.8	7.1
Fruit juices & drinks	54.8	385	752	8.2	18.5
Hard candies & mints	4.4	154	309	3.9	8.4
Non-herbal teas	30.3	590	1202	8.7	17.0
Sports drinks	2.2	360	749	6.4	13.2
Chewing gum	50.0	125	250	1.8	3.6
All categories	86.4	628	1284	11.3	24.2

Source: For all categories except chewing gum, 1994–96, 1998 Continuing Survey of Food Intakes by Individuals (USDA 2000). 90th percentile intakes from bottled water estimated at double mean intakes. For chewing gum, intakes based on data from Wm. Wrigley Jr. Company, assuming a log-normal distribution and a 70-kg consumer.

Of the food categories in which Suntheanine is intended to be used, fruit juices and drinks and chewing gum are by far the most widely consumed, by 50% or more of the United States population. Over 85% of the respondents to the CSFII reported consuming at least one of the target foods on at least one of the two survey days. On average, it is anticipated that individuals will consume 628 mg of Suntheanine per day (11.3 mg/kg bw/day). The EDI of Suntheanine under its intended conditions of use corresponds with the estimated 90th percentile of intake, or 1284 mg (24.2 mg/kg bw). These are roughly similar to the levels of L-theanine currently consumed by the heaviest tea drinkers in the United States, as was shown in Table 16.

V. Review of Safety Data

A. Safe Use of Glutaminase

As was discussed with regard to the production of Suntheanine, it results from the enzymatic synthesis of L-glutamine and ethylamine using glutaminase derived from either *Pseudomonas nitroreducens* or *Bacillus amyloliquefaciens*

Pseudomonas nitroreducens and *Bacillus amyloliquefaciens* are both ubiquitous soil bacteria that have never been implicated in human illness. The American Type Culture Collection (ATCC) classifies cultures by biosafety level based on assessment of the potential risk, using United States Public Health Service guidelines with assistance provided by ATCC scientific advisory committees (ATCC 2005). Both bacteria are classified as being at biosafety level 1, the least restrictive level, assigned to organisms not known to cause disease in healthy adult humans.

A total of 47000 kg of Suntheanine has been produced by enzymatic synthesis since 1994 with no reports of illness or discomfort by those handling *Pseudomonas nitroreducens* or *Bacillus amyloliquefaciens*, including all factory workers. Current annual production is approximately 30000 kg per year. Furthermore, there have been no reports of illness or discomfort by consumers of Suntheanine produced via *Pseudomonas nitroreducens* or *Bacillus amyloliquefaciens*.

As was discussed earlier with regard to the purity of Suntheanine, HPLC and spectrographic analyses have confirmed that no proteins or other foreign materials are present in Suntheanine; thus, neither fragments of the microorganism nor the enzyme are in the final product. Pariza and Johnson (2001) list both protease and the carbohydrase α-amylase from at least two different strains of *B. amyloliquefaciens* among enzymes used in food processing at the time the article was prepared; FDA has repeatedly confirmed the GRAS status of enzyme preparations from this bacterium (FDA 1999a, 1999b, 2003). Based on the above information and other information available in the scientific literature, an independent assessment of the safety of the use of glutaminase derived from either bacterium in the production of Suntheanine concluded that there is no need for further safety evaluation of the organisms or the enzyme (Pariza 2004).

B. Safe History of Consumption of Tea Containing L-Theanine

As was discussed in the previous section, after water, tea is the most widely consumed beverage in the world (Graham 1992; Blumberg 2003); over 2½ tons of tea leaves are produced annually (Chen et al. 2003). Even in the United States, not noted as a major tea-consuming country, over 20% of NHANES survey respondents age 13 and older and nearly 20% of respondents of all ages reported having consumed tea on the survey day. Their average consumption of tea on days when they drank it was nearly 800 g—about 28 ounces. Blumberg

(2003) estimated the average worldwide per capita daily intake of tea at about 120 ml; since this per capita intake includes infants and toddlers, as well as older non-tea drinkers, it is evident that consumption among older children, adolescents, and adults is much higher. Based on a 2003 world population of about 6.2 billion, per capita consumption of 120 ml of tea equates to daily consumption of nearly a million metric tons of tea. Despite this enormous level of consumption, tea has never been implicated in toxic or other adverse effects.

C. Safe History of Use of Dietary Supplements Containing Suntheanine

According to the manufacturer, Suntheanine is marketed in Asian markets in chewing gum, formulated beverages, bottled water, and chewable tablets, with Suntheanine concentrations ranging from 50–300 mg/serving. Suntheanine was introduced to the United States market as a dietary-supplement ingredient in 1999, and is available in hard capsules, gel capsules, chewable tablets, powders, beverages, and oral sprays containing concentrations of 100–300 mg/dose. Over 70000 kg of Suntheanine have been consumed since its initial launch in 1994, with no reports of adverse effects.

Schmidt et al. (2005) reported that reports of sporadic cases of liver disorders (acute hepatitis, icterus, hepatocellular necrosis) after ingestion of dietary supplements based on hydro-alcoholic extracts from green tea leaves have led to restrictions of the marketing of such products in certain countries of the European Union. However, Graham (1992) noted that tea leaves are about 36% polyphenols by weight and that they specifically contain a number of catechins, including catechin, epicatechin, gallocatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate. Schmidt et al. (2005) dosed rat hepatocytes in primary culture with (-)-epicatechin, (-)-epigallocatechin-3-gallate, caffeine, and theanine at concentrations reflecting their levels in a typical green tea extract. Cytotoxicity was found with (-)-epigallocatechin-3-gallate only; theanine was not observed to have cytotoxic effects.

Indeed, in a study using 5-week-old male Wistar rats, He et al. (2000) found that, far from having a hepatotoxic effect, theanine extracted from leaves of green tea had a suppressive effect on hepatotoxicity induced by D-galactosamine, shown by suppression of GalN-induced plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities.

D. Kinetics and Metabolism of L-Theanine

1. Absorption of Orally Administered Theanine

Kitaoka et al. (1996), in an *in vitro* study with everted sacs prepared from guinea pig ileum, investigated the mode of intestinal absorption of theanine by measuring the ionic dependency and kinetic properties of the theanine- and glutamine-evoked transmural electrical potential difference changes (Δ-PD) when they applied theanine and glutamine to the luminal side of the sacs. Both theanine and glutamine applied to the luminal side induced dose-dependent

increases in Δ-PD (increase in serosal positive value). The theanine- and glutamine-evoked Δ-PD maximum values were not significantly different, but the half-saturation concentration was much lower for glutamine (3.1 mM) than for theanine (21.4 mM). The theanine-evoked Δ-PD value was much smaller when theanine was applied in the presence of glutamine than when applied alone, and the theanine- and glutamine-evoked Δ-PD values were both inhibited by removing Na^+ from the luminal solution. The investigators interpreted their results as suggesting that the intestinal absorption of both theanine and glutamine is mediated by a common Na^+-coupled co-transporter in the brush-border membrane, the affinity of which is lower for theanine than for glutamine.

2. Distribution of Orally Administered Theanine

Using young male Wistar rats (90–110 g) divided into groups of six and gavaged with 4 g/kg bw theanine or saline, Terashima et al. (1999b) studied the distribution of theanine. The rats were killed by decapitation at 0, 1, 2, 4, 8, 16, or 24 hours after administration and blood, urine, and tissues were collected and analyzed for theanine concentrations.

After oral administration of theanine, theanine peaked in the blood at 1 hour, in the liver at 1–2 hours, and in the brain at 5–8 hours. By the end of 24 hours, no detectible theanine remained in the blood or tissues. The results show that theanine was rapidly incorporated into many tissues such as the blood, liver, and brain, and thereafter its levels were reduced within 24 hours, with a concomitant increase in the urinary excretion of theanine. Concentrations of the theanine metabolites glutamic acid and ethylamine were also increased in all tissues.

Theanine labeled with ^{14}C administered to rats intraperitoneally was taken up by brain tissue within 30 minutes (much more quickly than with oral administration) without any metabolic changes (Kimura and Murata 1971a). The effect of theanine on amino acids in the brain was investigated in a study in which rats were administered theanine via gavage (Yokogoshi et al. 1998a). This research showed that theanine is incorporated into brain tissue via a leucine-preferring transport system.

3. Metabolism of Orally Administered Theanine

Unno et al. (1999) measured plasma and urinary levels of both theanine and ethylamine, a known metabolite, after oral ingestion of theanine. Six-week-old male Wistar rats weighing 110–140 g received oral doses of 0, 100, 200, or 400 mg theanine in 2 ml water. Blood was drawn at 0, 15, 30, 60, 120, and 240 minutes after administration, and urine was collected for 24 hours. Plasma concentrations of both theanine and ethylamine increased rapidly after oral administration, theanine peaking at 30 minutes and ethylamine peaking at 2 hours. Plasma levels of both were dose-dependent.

HPLC chromatograms of the urine indicated distinct peaks due to the metabolites of theanine, and these were identified by the retention time of each compound as glutamic acid, theanine, and ethylamine, in that order. At doses of 100, 200, or 400 mg of theanine, the molecular percentages of theanine excreted into urine during 24 hours were 2%, 14%, and 21%, respectively, and those of ethylamine were 26%, 22%, and 21%, respectively.

In another part of the study, theanine was incubated *in vitro* with homogenates of tissue samples from the brain, heart, lung, liver, kidney, and spleen for metabolic study. Only the kidney homogenate was associated with the decomposition of theanine to ethylamine.

The investigators concluded that theanine is taken up into the blood circulation through the intestinal tract and then distributed to tissues and that the kidney is the site for the enzymatic hydrolysis of theanine. The maximum concentration of ethylamine in plasma after oral administration was much lower than that of theanine. If *in vitro* findings can be extended into the *in vivo* stage, the appearance of ethylamine in plasma can be explained by the metabolism of theanine in the kidney, with most of the ethylamine generated immediately excreted into the urine and only a part being recirculated in the plasma.

Tsuge et al. (2003) further investigated the enzymatic degradation of theanine *in vitro*. As enzyme sources, they prepared homogenates of brain, thymus, heart, lung, liver, muscle, spleen, small intestine, blood plasma, and kidney from Wistar rats. A solution consisting of 20 mM theanine in a total volume of 1.0 ml was added to each homogenate. After the reaction was stopped by adding HCl, the supernatant solution was withdrawn and the amount of L-glutamate released was determined. Theanine-degrading activity was detected exclusively in kidney homogenate. No theanine-degrading activity was found in the homogenates from other tissues. The kidney homogenate was purified, increasing the theanine-degrading activity about 600-fold from the crude kidney homogenate. Activity was detected from both isozymes, phosphate-dependent and phosphate-independent glutaminase. Activity of γ-glutamyl transpeptidase was also observed; this latter activity is possessed by phosphate-independent glutaminase only. Purified phosphate-dependent glutaminase did not show theanine-degrading activity at all.

Purified theanine-degrading enzyme preparation was subjected to polyacrylamide gel electrophoresis (PAGE), which indicated that the theanine-degrading enzyme has a molecular weight of about 70 kDa and is composed of two heterogeneous subunits, one weighing about 30 kDa and the other about 40 kDa. From the somewhat dispersed protein staining pattern of the native enzyme preparation, it was suggested that the enzyme might contain carbohydrate. After PAGE, the gel was stained with Coomassie brilliant blue R for protein and periodic acid-Schiff staining for carbohydrate, both of which were detected.

The authors concluded that their results confirm the findings of Unno et al. (1999) that theanine is metabolized only in the kidney. Furthermore, they have identified one of the renal glutaminase isozymes, phosphate-independent glutaminase, as the enzyme responsible for theanine metabolism, and determined that a large part of the theanine molecule must be converted to glutamyl peptides and ethylamine via γ-glutamyl transpeptidase reaction *in vivo*.

There is some indication from *in vivo* work with male C57BL/6 and BDF(1) mice that theanine may also undergo metabolism in the liver, producing glutamate as one metabolite (Sugiyama et al. 2003). This is based on a finding that the glutamate concentration in the liver increased after intraperitoneal administration of theanine, supported by an *in vitro* phase in which liver tissue was homogenized in the presence or absence of theanine, and theanine significantly enhanced (by about 50%) glutamate generation.

4. Excretion of Orally Administered Theanine

Several of the studies discussed above that investigated the absorption, distribution, and metabolism of orally administered theanine also provided information on excretion. Kitaoka et al. (1996) found that not all ingested theanine is absorbed, but they did not determine whether the unabsorbed portion is degraded by colonic microflora or excreted unchanged in the feces. Similarly, Desai et al. (2005), in a study of the relative kinetics of the two theanine enantiomers (discussed more fully below), reported that D-theanine is less well absorbed than is L-theanine but did not investigate the fate of the unabsorbed material.

Terashima et al. (1999b) found that theanine was no longer present in plasma or in the brain or other tissues 24 hours after oral administration, but that the level of theanine in the urine showed a rise matching the decline in the theanine concentrations in plasma and tissue. Unno et al. (1999) found that a portion of the absorbed theanine was metabolized into ethylamine in the kidneys and that substantial fractions of both the absorbed theanine and its metabolite ethylamine were excreted in the urine. Desai et al. (2005) found that theanine was gone from the plasma within 6 hours of administration, although ethylamine was not, and that urinary concentrations of theanine reached their maximums 1–4 hours after administration.

5. Comparison of the Metabolism and Kinetics of L-Theanine and D-Theanine

As has been noted, Suntheanine is pure L-theanine; the D- enantiomer is not present at detectible levels (Desai and Armstrong 2004, Armstrong undated). Desai et al. (2005) pointed out that the chiral nature of theanine implies that the two enantiomers may have different activities. They dosed male Sprague-Dawley rats weighing 300–500 g with L-theanine, D-theanine, or a racemic mixture, either via gavage or intraperitoneally (i.p.). Blood was taken from the lateral saphenous vein at 0, 15, 30, 60, 120, 240, and 360 minutes after administration, and urine samples were taken at 0, 60, 120, 240, and 360 minutes after administration. Both were analyzed for the concentrations of L- and D-theanine. Because theanine is metabolized in the kidney to glutamic acid and ethylamine (Tsuge et al. 2003), ethylamine concentrations in the plasma were also analyzed.

With oral administration of pure L-theanine or D-theanine, plasma concentrations of the L- enantiomer were more than three times the level of D-theanine. This difference was dose-dependent—at lower doses, the plasma concentration of L-theanine was nearly five times that of D-theanine. The maximum plasma concentrations were reached 30–60 minutes after administration for both enantiomers at all tested doses. The racemic mixture produced higher concentrations of L-theanine than of D-theanine. Peak concentrations were achieved a little later than with the single-enantiomer doses, about 60 minutes after administration. The maximum plasma level of the D-enantiomer from the racemic mixture was three times lower than with administration of D-theanine alone. Theanine had essentially disappeared from the plasma within 6 hours, but the ethylamine concentration did not diminish over the 6-hour experiment after administration of either enantiomer.

Urine concentrations peaked at 1–2 hours for racemic and pure L-theanine and at 2–4 hours for D-theanine. More D-theanine was found in the urine 2 hours after administration of the racemic mixture than from administration of D-theanine alone, but the total area under the curve

(AUC) was similar. Similarly, more L-theanine was found in the urine from the racemic mixture than from administration of L-theanine alone. The rats did not experience diuresis due to theanine administration, either of the single enantiomers or of the racemic mixture.

With i.p. administration, maximum plasma levels were observed at 15–30 minutes post administration. The total AUC for L-theanine administered by i.p. was not different from that found with oral administration, but D-theanine's AUC was significantly higher when administered via i.p. and was similar to that for L-theanine. The plasma AUCs for the racemic mixture were lower than when either the D- or L- enantiomer was administered alone. The urinalysis found that more D-theanine than L-theanine (6400 v. 860 μg/ml) was excreted after i.p administration of the racemate.

The investigators concluded that with oral administration, L-theanine was better absorbed than was D-theanine (i.e., there was chiral discrimination in absorption). Maximum levels in the plasma were strictly dose-dependent, with no sign of a leveling off, and thus no apparent saturation. Absorption of both D- and L-theanine was lower from the racemate than from administration of a single enantiomer, but the plasma-concentration/time profile did not differ, indicating comparable kinetic profiles. However, D-theanine from the racemate was eliminated more slowly than from administration of the single enantiomer.

Since the plasma concentration of L-theanine was similar from oral and i.p. administration but the concentration of D-theanine was much lower with oral administration than with i.p., Desai et al. (2004) concluded that "the gut plays a major role in inhibiting D-theanine uptake." They also suggested that this stereoselectivity implies an active transport, rather than passive diffusion.

Absorption of D- and L-theanine from the racemate shows that there is competition for absorption between the D- and L- enantiomers, indicating a common transporter across the brush-border membrane. Intraperitoneal administration, however, was not better than oral administration for racemic theanine, indicating that renal reabsorption also plays a regulatory role.

6. Toxicokinetics of Suntheanine

A 90-day oral toxicity study of Suntheanine in the rat (Borzelleca et al. 2006), discussed in more detail in the following section on toxicological studies, included a toxicokinetic segment. The doses of Suntheanine in this study were 1500, 3000, and 4000 mg/kg bw/day. Plasma samples were collected on one day during week 3 at18:00 and on the following day at 00:00, 03:00, 06:00, 12:00, and 18:00; this schedule of sampling was repeated during week 13. The maximum concentrations of L-theanine in the plasma increased as the dose levels increased (Figure 3), as did the total AUC (Figure 4). For most groups, the time of the maximum concentration fell within the dark cycle, which is consistent with the eating habits of rodents.

Figures 3 and 4 also clearly show that there was no accumulation of L-theanine in the plasma of the rats between weeks 3 and 13 at any dose level; neither the maximum L-theanine concentrations nor the AUCs are significantly different between the two testing periods.

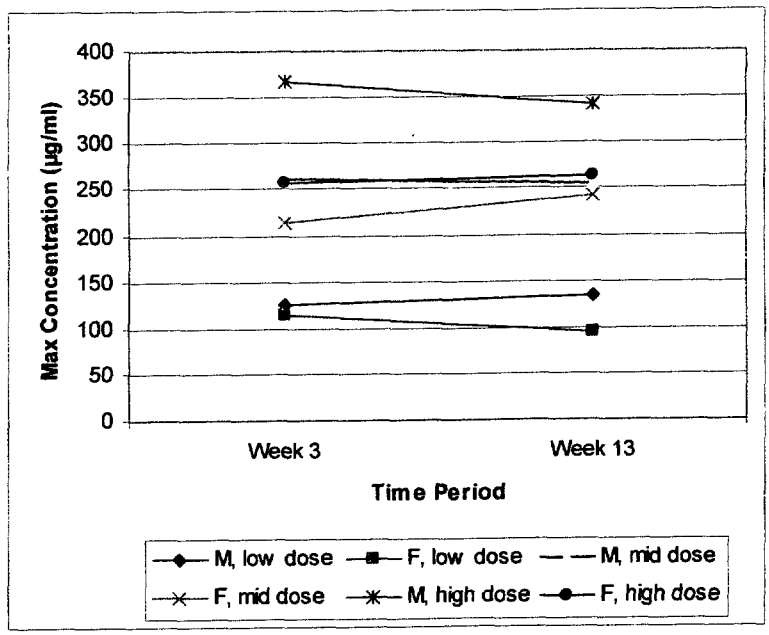

Figure 3. Maximum Plasma Concentrations of L-Theanine

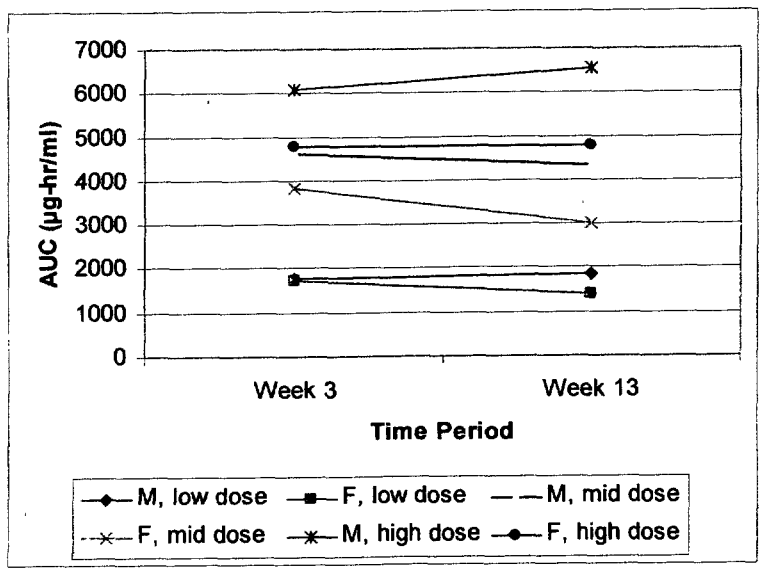

Figure 4. L-Theanine Plasma Concentration Areas Under the Curve

E. Toxicological Studies of Suntheanine

Toxicological studies demonstrating the safety of L-theanine have been conducted in rodents. These include an acute oral study, a 28-day study, two 13-week studies, and a 78-week study. In addition, an Ames assay and an *in vitro* cytogenetics assay were conducted. These studies are summarized below.

1. Acute Study

Following one week of acclimatization, groups of 4-week-old Wistar rats (7–8 rats/sex/group) weighing approximately 70 g were orally administered a single dose of 2500 or 5000 mg Suntheanine/kg bw dissolved in 0.1 ml saline/10 g bw (Taiyo Kagaku Research Center 1999). Control rats (7 rats/sex/group) received 0.1 ml saline/10 g bw. Rats were housed individually and observed for 7 days. Body weights were measured daily. One female rat in the high-dose group died one day after treatment; however, the death was attributed to accidental dislocation of the neck. No other effects were reported, and there were no statistically significant differences in body weight between treated and corresponding control animals. The oral LD50 of Suntheanine was determined to be >5000 mg/kg bw.

2. Subacute Study

In a 28-day study, Crj:CD(SD) rats (5 rats/sex) weighing 73–84 g were gavaged with 2000 mg Suntheanine/kg bw/day dissolved in distilled water (Nippon Bio Research Center Co. 1999a). Control rats (5 rats/sex) received 10 ml distilled water/kg bw. The rats were housed individually and observed daily before and after treatment. Body weights were measured twice per week. Feed consumption over a 48-hour period was determined weekly, and daily feed consumption was calculated from the 48-hour results. Ophthalmological examinations were conducted prior to treatment and during the last week of treatment. Urine samples were collected during the last week of treatment over a 24-hour period. For the initial 3 hours, feed was withheld, but the rats had access to water. For the remaining 21 hours, rats had access to both feed and water. The urine samples (first 3 hours, next 21 hours, and total urine for 24 hours) were analyzed for color, occult blood, pH, protein, sugar, ketone bodies, urobilinogen, and bilirubin. Blood samples were collected from the abdominal aorta of all rats one day after the last treatment and were analyzed for red blood cells, hemoglobin, hematocrit, platelet, white blood cells, mean corpuscular volume, mean concentration of hemoglobin, percent white blood cells, and reticulocyte count. In addition, serum samples were analyzed for aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total cholesterol, triglycerides, total protein, urea nitrogen, creatinine, total bilirubin, glucose, inorganic phosphorus, calcium, sodium, and potassium. After the blood samples were collected, the rats were euthanized, and gross post-mortems were conducted. The following organs were removed and weighed: pituitary gland, thyroid glands, brain, salivary glands, thymus, lungs, heart, liver, spleen, kidneys, adrenal glands, testes, prostate, ovaries and uterus. Samples of heart, aorta, lung, bronchus, liver, pancreas, tongue, salivary glands, stomach, duodenum, jejunum, ileum, cecum, rectum, thymus, spleen, lymph nodes, kidneys, bladder, testes, epididymis, seminal vesicles, prostate, ovaries, uterus, vagina, mammary glands, pituitary gland, adrenal glands, thyroid gland, parathyroid gland, brain, spinal cord, skin, eye, Harderian gland, bone, and bone marrow were prepared and examined microscopically. Results were statistically analyzed by F-test and, if necessary, by Student's t-test or Aspin-Welch t-test.

All rats survived to study termination, and no adverse clinical signs were reported. Body weights and feed consumption were similar between treated and corresponding control groups. Ophthalmological examination revealed no abnormalities in the anterior chamber, eyeball, eyelid, conjunctiva, sclera, iris, cornea, or fundus in any of the rats. Urinalysis showed no

significant difference between treated and control groups in the parameters tested. The only statistically significant changes in hematological parameters studied were an increase in fibrinogen (control: 250.8 ± 5.2 mg/dl; treated: 271.4 ± 9.0 mg/dl) and decrease in platelets (control: 118.02 ± 6.23x10^4/mm^3; treated 108.62 ± 2.29x10^4/mm^3) in treated male rats compared to controls. No changes were reported in females. Similarly, the only statistically significant changes in biochemical parameters were seen in treated males: increased alkaline phosphatase (control: 186.34 ± 74.51 IU/l; treated: 283.00 ± 48.10 IU/l) and increased β-globulin in the protein fraction (control: 11.10 ± 0.48%; treated: 11.94 ± 0.59%) compared to controls.

Upon gross pathological examination, no abnormalities were reported. No statistically significant differences in absolute or relative organ weights were reported between treated females and controls. In treated males, absolute brain weight (control: 1.998 ± 0.041 g; treated: 1.884 ± 0.072 g), but not relative brain weight, was significantly lower than that of controls and both absolute (control: 14.390 ± 0.743 g; treated: 16.334 ± 1.311 g) and relative liver weights (control: 3.932 ± 0.269 g%; treated: 4.374 ± 0.149 g%) were significantly higher than those of controls. Histopathologically, the treated males showed no changes in the brain or liver; however, slight myocardial denaturation was found in the hearts of two rats, and slight lymphocyte infiltration in the stroma of the kidney and prostate of one rat was reported. In females, one control animal had slight bleeding in the thymus, one treated rat had slight mineral deposition in the stomach, one treated rat had slight heterotopia thyroid, and another treated rat had a vestigial postbrachial body in the thyroid. Based on their extent and frequency, the histological results were considered to be spontaneous findings and not evidence of toxicity. The NOAEL in this study was the only dose tested, 2000 mg/kg bw/day.

3. Subchronic Studies

a. Mice

A 13-week dietary study in mice was conducted to determine the maximum tolerated dose (MTD) of L-theanine (Fujii et al. 1999). Groups of B6C3F1 mice (10/sex/group) weighing 16-26 g were fed diets containing 0 (control), 0.6, 1.25, 2.5, or 5% L-theanine (approximately 0, 850, 1700, 3150, or 6300 mg/kg bw/day, respectively, for males and 0, 550, 1450, 2550, or 5150 mg/kg bw/day, respectively, for females). Animals were housed in groups by sex. Body weights were taken one week after treatment commencement, and feed consumption was determined weekly. The day after the last dosing, animals were terminated and necropsied. Heart, lung, submandibular gland, esophagus, stomach, small intestine, large intestine, liver, pancreas, kidneys, testes, ovaries, thyroid gland, adrenal glands, thymus, spleen, bone marrow, brain, and spinal cord were microscopically examined.

All the mice survived to study termination. Feed consumption was similar among all the groups. All treated animals had body weights within 10% of those of the corresponding controls. No pathological changes were reported. The MTD of L-theanine was considered to be the highest dietary concentration tested of 5%, approximately 6300 mg/kg bw/day for males and 5150 mg/kg bw/day for females.

b. Rats

Groups of 7-week-old male and female Crl:CD®(SD)IGS BR rats (20/sex/group) were fed diets containing L-theanine at concentrations providing 0 (control), 1500, 3000, or 4000 mg/kg bw/day for 13 weeks (Borzelleca et al. 2006). The study was conducted following test guidelines promulgated by OECD (408) and FDA (FDA 1993) for toxicity testing and GLP. The individually-housed rats were observed twice daily for clinical signs, and detailed clinical examinations were made weekly. Behavioral observations were conducted on 10 rats/sex/group once during the pretest and once during weeks 3 and 13. Motor tests were conducted on the same animals prior to the study and during weeks 3 and 14 or 15. Ophthalmological examinations were performed prior to study commencement and during week 13. Body weights were measured prior to study commencement, weekly thereafter, and prior to termination. Feed consumption was determined weekly. Blood samples were drawn on day 30 or 31 and at termination from the jugular veins of unanesthetized rats, which were fasted overnight, for hematological and biochemical analysis. Urine samples were collected before blood collection. After 90 days of treatment, the rats were fasted overnight, blood samples were taken, and the rats were weighed, anesthetized with sodium pentobarbital, exsanguinated, and necropsied. During necropsy, the carcasses were grossly examined, organ weights were taken, and tissues were removed and preserved for microscopic examination. Statistical analysis was conducted using Levene's test, one-way ANOVA, and Dunnett's t-test.

No consistent, remarkable treatment-related clinical observations and no treatment-related deaths were reported. Five animals died: two due to cystitis, two due to complications from blood collection, and one (sacrificed) due to respiratory distress from a severe malocclusion. Body weights of both sexes were significantly lower than controls at the two highest doses; however, this was related to significantly reduced feed consumption as a result of poor palatability and thus is not regarded as a toxic effect. During a few weekly intervals, feed efficiency was lower in the mid- and high-dose males and females than in controls. However, this effect was not consistent and overall feed efficiencies did not differ among the groups. No adverse effects on behavior and motor activity related to treatment were reported. No consistent, statistically significant, dose-dependent, treatment-related adverse effects were reported in any of the ophthalmological, hematological, clinical chemical or urinalysis parameters examined. The only treatment-related effects reported were limited to mildly lower total protein values in high-dose females, mildly higher cholesterol values in females administered 3000 or 4000 mg L-theanine/kg bw/day, and mildly higher urine pH in males administered 3000 or 4000 mg L-theanine/kg bw/day. None of these findings was considered adverse or indicative of significant target organ toxicity. Significant dose-dependent increases in kidney weights (absolute, relative to body weight, and/or organ-to-brain ratio) were observed in both sexes. Significant dose-dependent decreases in thymus weights (absolute, relative to body weight, and/or organ-to-brain ratio) were reported in males. These organ-weight differences were not clearly adverse, since there were no histological correlates and there were no clinical pathological indicators of organ toxicity. At necropsy, the only findings were renal tubular cell adenomas in two high-dose and one mid-dose females and renal tubular cell hyperplasia in these same three females but no others administered 3000 or 4000 mg L-theanine/kg bw/day. No renal lesions were observed in any other high- or mid-dose females or in any low-dose females or in any of the males. The renal findings were not consistent with the characteristics of a renal carcinogen due to the early onset yet low number of affected animals, but appeared to be more consistent with a genetic

predisposition rather than with a direct toxic effect. In order to fully understand these findings, an intensive investigation was undertaken. The results of this investigation, published separately (Hall et al. 2006), led to the following conclusion:

> "The tumors were papillary cystadenomas, multicentric and bilateral, and accompanied by atypical foci of renal tubular hyperplasia in both kidneys of the three animals. Toxic tubular changes that typically accompany renal carcinogenesis were not seen in any of the other animals of the study as verified by step-sectioning the kidneys of all female rats. The presence of bilateral, multi-focal tumors in animals in the absence of any toxic tubular changes suggested the possibility of an underlying germline mutation in a tumor suppressor gene rather than compound related carcinogenesis. The histological appearance of these tumors and short latency were reminiscent of the spontaneous lesions reported to arise in Sprague Dawley rats in the Nihon rat model. Nihon rats develop kidney tumors as a result of a spontaneous mutation in the rat homologue of the Birt-Hogg-Dubé gene (*Bhd*). Frozen samples of liver from two tumor-bearing rats were assayed for germline alterations in the *Bhd* gene. The entire coding region (exons 3-13) of the *Bhd* gene was sequenced, and a guanine ($nt106^{G}$) to adenine ($nt106^{A}$) polymorphism was detected resulting in a glycine-to-arginine (G36R) substitution in both tumor-bearing animals. In the study animals, the frequency of the A-allele (adenine) was determined to be 27% (19/70). Interestingly, rats obtained from two other sources (n=17) only carried the $nt106^{G}$-allele, consistent with the published rat sequence for this gene. Genetic fingerprinting of microsatellite loci indicated that the rats had a shared genetic background. Laser capture microdissection (LCM) of the tumor cells from one of the rats demonstrated a loss of heterozygosity in the *Bhd gene* in neoplastic cells. Taken together, these data suggest that the tumors observed in these animals arose spontaneously as a result of a shared genetic susceptibility resulting in mutation of the *Bhd* gene and development of renal tumors. These events were not considered test article-related" (Hall et al. 2006)

Based on the absence of consistent, dose-dependent, test-article-related adverse effects, it was concluded that the NOAEL in this study was the highest dose tested, 4000 mg/kg bw/day (Borzelleca et al. 2006).

4. Chronic Study

In a follow-up study to the 13-week mouse study (see above), groups of male and female B6C3F1 mice (50/sex/group) weighing 16-23 g were fed diets containing 0 (control), 3, or 5% L-theanine (approximately 0, 2200, or 4400 mg/kg bw/day, respectively, for males and 0, 2000, or 4150 mg/kg bw/day, respectively, for females) for 78 weeks (Fujii et al. 1999). Animals were housed five per cage. Body weights and feed consumption were determined weekly for the first 4 weeks and every 4 weeks thereafter until study termination. One day following the last dose, surviving animals were killed and necropsied.

The number of surviving animals [males: 43 (control), 41 (2.5%), and 45 (5%); females: 47 (control), 46 (2.5%), and 48 (5%)] and mean survival period were similar among all groups. In females, final body weights tended to be lower than those of control animals; however, there were no statistically significant differences in mean body weight or final body weights between treated mice of either sex and corresponding control mice. Feed consumption was similar among all the groups. Necropsy results showed no significant differences in lesion and tumor incidences. In fact, treated mice tended to have a lower incidence of lung and liver tumors than controls. Administration of up to 5% L-theanine in the diet of mice for 78 weeks showed no evidence of carcinogenicity.

5. Genotoxicity/Mutagenicity Studies

Suntheanine was tested using the preincubation method Ames assay in *Salmonella typhimurium* strains TA100, TA98, TA1535, and TA1537 and *Escherichia coli* strain WP2*uvrA* at concentrations of 0 (sterile water negative control), 50, 100, 500, 1000, and 5000 μg/plate with and without metabolic activation (S9) (Nippon Bio Research Center Co. 1999b). The test substance was diluted using sterile water. The positive controls used were 2-aminoanthracene (all strains with S9), sodium azide (TA1535 without S9), 9-aminoacridine hydrochloride (TA1537 without S9), and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (TA100, TA98, and WP2*uvrA* without S9). No precipitation or growth inhibition was seen in any of the test groups at any concentration tested. The number of revertant colonies in all Suntheanine-treated cultures was less than two times that of the negative controls. Positive controls showed a "remarkably higher" revertant colony count than that of the negative controls. Suntheanine was not mutagenic in this assay.

L-theanine also was tested in an *in vitro* cytogenetics assay using duplicate human lymphocyte cultures under GLP and following OECD guideline 473 (Lloyd 2004). L-theanine was diluted with water and tested in two independent experiments at various concentrations with and without S9 mix prepared from the liver of male Sprague-Dawley rats induced with Aroclor 1254. The negative control used was sterile purified water, and the positive controls used were mitomycin C (without S9) and cyclophosphamide (with S9).

In the first experiment, lymphocyte cultures were treated for 3 hours at concentrations of 3200, 4000, or 5000 μg/ml followed by a 17-hour recovery period. The highest concentration induced about 15% and 13% mitotic inhibition with and without S9, respectively. In the second experiment, lymphocyte cultures were treated without S9 for a continuous 20 hours or with S9 for 3 hours followed by a 17-hour recovery period. The highest concentration induced about 14% and 0% mitotic inhibition with and without S9, respectively. In both experiments, the positive controls induced a statistically significant increase in the proportion of cells with structural aberrations when analyzed 20 hours after the start of treatment. The frequency of cells with structural aberrations was similar between L-theanine-treated cultures and concurrent negative controls. L-theanine did not induce chromosome aberrations in human peripheral blood lymphocyte cultures with or without metabolic activation.

VI. Safety Assessment and GRAS Determination

A. Introduction

This chapter presents an assessment that demonstrates that Suntheanine is safe, and is GRAS, for direct addition to foods to increase the dietary intake of L-theanine.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of Suntheanine under its intended conditions of use is demonstrated. Safety is established by demonstrating that the EDI of Suntheanine under its intended conditions of use is within allowable levels of intake. In the second step, Suntheanine is determined to be GRAS by demonstrating that the safety of this product under its intended conditions of use is generally recognized among qualified scientific experts, and is based on publicly available and accepted information.

The regulatory framework for establishing whether a substance is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedures GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components:

1) data and information relied upon to establish the scientific element of safety must be generally available; and

2) there must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific-procedures GRAS determination are applied below in an analysis of whether Suntheanine, used as a nutrient supplement, is safe, and is GRAS.

B. Safety of Suntheanine

A scientific procedures GRAS determination requires that information about the substance establish that the intended use of the substance is safe. The FDA has defined "safe" or "safety" for food additives under 21 CFR §170.3(i) as "a reasonable certainty in the minds of competent scientists that the substance is not harmful under its intended conditions of use." This same regulation specifies that three factors must be considered in determining safety. These three factors are:

1) The probable consumption of the substance and of any substance formed in or on food because of its use (i.e., the EDI);

2) The cumulative effect of the substance in the diet, taking into account any chemically- or pharmacologically-related substance or substances in such diet; and

3) Safety factors, which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food and food ingredients, are generally recognized as appropriate.

1. EDI of Suntheanine

As was discussed in detail in Chapter IV, Suntheanine is intended to be added to foods in a number of different food categories to provide up to 250 mg/serving of L-theanine. The food categories in which use of Suntheanine is intended include fruit juices and drinks, non-herbal teas, sports beverages, specialty bottled waters, chocolate bars and chews, hard candies and breath mints, and chewing gum. Over 85% of Americans age 2 or older are users of at least one of these categories of food, and the mean daily intake of Suntheanine from these intended uses is 628 mg/day, or 11.3 mg/kg bw/day. The EDI, corresponding to the 90th percentile of intake of Suntheanine, is 1284 mg/day, or 24.2 mg/kg bw/day.

2. Establishing the Safety of Suntheanine

It was noted in Chapter IV that tea is the only naturally occurring source of theanine in the diets of Americans and, in fact, of most people of the world. After water, tea is the most widely consumed beverage in the world. Even in the United States, where tea competes with coffee and numerous other beverages, it is consumed by many adults and children. In the 1999–2002 NHANES, about 20% of adult respondents reported having consumed tea on the survey day. The mean amount of tea consumed by these individuals was 763 g, but the top 10% of consumers drank 1650 g of tea, and the top 1% of consumers drank 3337 g. It is probable that many tea-drinkers in more tea-intensive countries may consume tea at these or even higher levels.

The theanine content of tea leaves is generally in the range of 1–2.5%; since theanine is highly soluble in water, nearly all of the theanine in the leaves is likely to dissolve in the water when a tea beverage is prepared. The estimated intake of theanine—nearly all of it L-theanine—by tea drinkers was estimated as 152–382 mg/day at the mean, and as much as 667–1668 mg/day

at the 99th percentile. This last range encompasses the level of intake estimated to result from the intended uses of Suntheanine.

Metabolism and toxicokinetic studies have shown that the intestinal absorption of L-theanine is mediated by a Na^+-coupled co-transporter in the brush-border membrane. After absorption, theanine is rapidly incorporated into the blood and many tissues such as the liver and brain, this last via a leucine-preferring transport system. Theanine is hydrolyzed to glutamine and ethylamine, primarily in the kidneys but possibly also in the liver. Much of the metabolite is immediately excreted, but some returns to the plasma. Both theanine and its metabolites reach peak concentrations in the plasma and in tissues within a few hours then rapidly decrease, along with a concomitant increase in urinary concentrations. The evidence available shows that theanine does not accumulate in plasma but is rapidly excreted.

The oral LD50 of Suntheanine is >5000 mg/kg, showing a low order of oral toxicity. A subacute (28-day) study obtained an oral NOAEL at the only dose tested, 2000 mg/kg bw/day. In a 13-week subchronic dietary study, rats were dosed with 1500, 3000, or 4000 mg Suntheanine/kg bw/day. No toxicity was found at any tested dose, and the NOAEL was the highest dose tested, 4000 mg/kg bw/day. A 78-week chronic study of Suntheanine in mice found no evidence of carcinogenicity and genetic assays have shown that it is not mutagenic.

C. General Recognition of the Safety of Suntheanine

The proposed uses of Suntheanine in food have been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b). This safety was established by first establishing the identity and purity of the material and then estimating potential human exposure to Suntheanine from its intended uses in food. Finally, because this safety assessment satisfies the common knowledge requirement of a GRAS determination, this intended use can be considered GRAS.

Determination of the safety and GRAS status of Suntheanine for direct addition to foods under its intended conditions of use has been made through the deliberations of an Expert Panel consisting of Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., Robert J. Nicolosi, Ph.D., and Michael W. Pariza, Ph.D. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have critically reviewed and evaluated the publicly available information summarized in this document, including the potential human intake resulting from the intended use of Suntheanine, and have individually and collectively concluded:

> Suntheanine has been sufficiently characterized to ensure a food-grade product, and to ensure that no toxicity concerns from impurities exist. Ingestion of Suntheanine from the proposed uses results in intakes of Suntheanine that remain well within safe limits established by the history of widespread consumption of tea containing naturally occurring theanine and corroborated by published animal and human studies. Therefore, Suntheanine, meeting the specifications described

in this GRAS monograph, to be used as a nutrient supplement, is safe, and GRAS by scientific procedures.

It is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available data would reach the same conclusion. Therefore, Suntheanine is safe, and GRAS by scientific procedures, for the proposed uses described herein.

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CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF SUNTHEANINE® L-THEANINE IN FOOD

Prepared for:

Taiyo International, Inc.
Minneapolis MN

Taiyo Kagaku Co., Ltd.
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July 2005

CONCLUSION OF THE EXPERT PANEL: GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF SUNTHEANINE® L-THEANINE IN FOOD

We, the members of the expert panel, have individually and collectively critically evaluated the publicly available information on Suntheanine® and L-theanine summarized in a monograph prepared by JHEIMBACH LLC, as well as other material deemed appropriate or necessary. Our evaluation included review of the starting materials and methods of manufacture of Suntheanine®; kinetics and metabolism of L-theanine; genetic, acute, subacute, subchronic, and chronic toxicity of the product; and safety of increasing the L-theanine intake of the U.S. population to the extent anticipated by the intended use of Suntheanine®. Our summary and conclusion resulting from this critical evaluation are presented below.

Summary

- The substance that is the subject of this generally recognized as safe (GRAS) determination is Suntheanine®, comprising >98% L-theanine, an amino acid found in free form in tea (*Camellia sinensis*), where it constitutes about 1.0 to 2.5% of the weight of the tea leaf.

- Suntheanine® is produced by a novel enzymatic synthesis of L-glutamine and ethylamine by glutaminase derived by *Pseudomonas nitroreducens*, a ubiquitous soil bacterium with no known association with human illness. The product is enantiomerically pure, with no detectable levels of D-theanine and with no detectable residues of glutaminase.

- Multiple lots of Suntheanine® have been analyzed to demonstrate that the product is consistently produced in compliance with the specifications that have been established to ensure material is food-grade and of appropriate purity for human ingestion. Suntheanine® has been shown to be stable at 121°C for 15 minutes, at pH ranges of 3.0 to 7.0 for 4 months, and at room temperature (25°C) in closed containers for 2 years, ensuring that it remains a safe food-grade product over its shelf life of 18 months.

- The addition of Suntheanine® to foods is intended to increase the dietary intake of L-theanine. It is intended to be added to bottled water, ready-to-drink teas, sports drinks, fruit juices and drinks, chocolate bars and chews, hard candies and mints, and chewing gum at a level not to exceed 250 mg/serving.

- The estimated daily intake (EDI, corresponding to the 90th percentile of intake) of L-theanine from the intended use of Suntheanine® is 960 mg, or 21.1 mg/kg bw. This level of L-theanine intake is within the range currently ingested by heavy tea drinkers in the United States and worldwide who consume tea over their lifetimes.

- Studies of the kinetics and metabolism of L-theanine suggest that absorption is mediated by a Na^+-coupled transporter in the brush-border membrane and widely distributed to tissues, including the brain, where it is incorporated via a leucine-preferring transport system. It is rapidly decomposed to ethylamine and glutamate, predominantly in the kidneys, and excreted such that it is not detectible in blood plasma 6 hours after oral administration.

- The oral toxicity of Suntheanine® is very low; the LD_{50} in rats is >5000 mg/kg bw, the highest dose (limit dose) tested. The no observed adverse effect level (NOAEL) in a 28-day study in rats was 2000 mg/kg bw/day, the limit dose tested. In a 90-day study in rats the NOAEL was the highest dose tested, 4000 mg/kg bw/day. Administration of up to 5% Suntheanine® in the diet of mice for 78 weeks showed no evidence of carcinogenicity. Suntheanine® was not mutagenic in two assays at the limit concentrations tested.

- The metabolism of L-theanine follows well understood metabolic pathways and neither L-theanine nor its metabolites bioaccumulate.

- The EDI of 21.1 mg/kg bw/day resulting from the intended use of Suntheanine® is well within levels of intake that have been shown to be safe..

Conclusion

We, the undersigned expert panel members, have individually and collectively critically evaluated the materials summarized above.

We conclude that L-theanine (Suntheanine®) has been sufficiently characterized to ensure that it is a food-grade product and that no toxicity concerns from impurities exist. Ingestion of Suntheanine® from its intended use results in a level of intake that is within safe limits established by the history of consumption of L-theanine in tea and by published animal toxicity studies. Therefore, Suntheanine® complying with the specifications and use described in the GRAS monograph is safe for addition to food.

It is also the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusion. Therefore, Suntheanine® is safe, and is GRAS for its intended use via scientific procedures.

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Richmond, Virginia

Signature _____ Date: 01 July 2005

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Signature: _____ Date: 7/01/05

Michael W. Pariza, Ph.D.
Director, Food Research Institute
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Signature: _____ Date: 7/01/05

Determination of the GRAS Status of Suntheanine® L-Theanine for Use in Food

Addendum I: Alternative Source of Glutaminase

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Prepared by
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Washington DC

February 2006

Determination of the GRAS Status of Suntheanine® L-Theanine for Use in Food

Addendum I: Alternative Source of Glutaminase

Background

Suntheanine® is a branded product that is entirely composed of the amino acid L-theanine (N-ethyl-L-glutamine or L-glutamic acid-γ-monoethylamide). It is produced by the enzymatic synthesis of L-glutamine and ethylamine using glutaminase. In June of 2005, the product was determined to be generally recognized as safe (GRAS) for addition to fruit juices and drinks, ready-to-drink teas, sports beverages, specialty bottled waters, chocolate bars and chews, hard candies and breath mints, and chewing gum to provide up to 250 mg/serving of L-theanine (JHeimbach 2005, Borzelleca et al. 2005).

The glutaminase used in the synthesis of the GRAS product is derived from *Pseudomonas nitroreducens*, developed by Iizuka and Komagata (1964) and identified by the Institute for Fermentation, Osaka, Japan, as IFO 12694 and by Ajinomoto Co. Inc., Kawasaki, Japan, as AJ2282. The American Type Culture Collection designation of this bacterium is ATCC 33634.

Taiyo Kagaku Co., Ltd., the maker of Suntheanine, intends to also to synthesize the product using glutaminase derived from a different bacterium, *Bacillus amyloliquefaciens*, a member of the heterogeneous *Bacillus subtilis* group.

Safety of *Bacillus amyloliquefaciens* As a Source of Glutaminase

Like *Pseudomonas nitroreducens*, the *B. subtilis* group are ubiquitous soil bacteria; the group have been characterized by the Food and Drug Administration (FDA) as having no pathogenic potential to humans (FDA 1999a). The American Type Culture Collection (ATCC) classifies cultures by biosafety level based on assessment of the potential risk, using United States Public Health Service guidelines with assistance provided by ATCC scientific advisory committees (ATCC 2005). The *B. subtilis* group, including *B. amyloliquefaciens*, is classified as biosafety level 1, the least restrictive level, assigned to organisms not known to cause disease in healthy adult humans.

In granting *B. subtilis* exemption from review under the Toxic Substances Control Act, the Environmental Protection Agency (EPA) noted that it is "one of the most widely used bacteria for the production of enzymes and specialty chemicals" and that risks associated with its use are low (EPA 1997).

Two enzyme preparations derived from *B. subtilis* bacteria, specifically including *B. amyloliquefaciens*, are affirmed as GRAS for use in food production, carbohydrase (21 CFR 184.1148) and protease (184.1150). In affirming the GRAS status of these preparations, FDA noted that they were commonly used in food prior to 1958, and that this evidence is "corroborated by information that the enzymes themselves and the

sources from which they are derived are nontoxic and nontoxicogenic, and that manufacturing will not introduce impurities that would adversely affect the safety of the finished enzyme preparations" (FDA 1999a, p. 19893).

Additionally, FDA has responded to two GRAS notices for enzyme preparations derived from *B. subtilis*. Enzyme Bio-Systems Ltd. determined that a pullulanase preparation derived from a genetically modified *B. subtilis* is GRAS for use in the production of corn sweeteners, baked goods, and alcoholic beverages (GRN 000020); FDA had no questions regarding this determination (FDA 1999b). In GRN 000114, Japan Cellfoods Co., Ltd., informed FDA that it had determined that pectate lyase enzyme preparation from *B. subtilis* is GRAS for use in fruit and vegetable purees and concentrates; FDA had no questions regarding this determination (FDA 2003).

Pariza and Johnson (2001) list both protease and the carbohydrase α-amylase from at least two different strains of *B. amyloliquefaciens* among enzymes used in food processing at the time the article was prepared

Identification and Characterization of *Bacillus amyloliquefaciens*

The *B. amyloliquefaciens* strain intended for use as an alternate source of glutaminase is designated GT2. In 16S ribosomal nucleotide sequence analysis, it fully matches *B. subtilis* strain ATCC 9799. Since the species *B. subtilis* is rather heterogeneous, DNA:DNA hybridization was performed. This analysis revealed 95% DNA homology with ATCC 23350, the type strain of *B. amyloliquefaciens* (Priest et al. 1987). *B. amyloliquefaciens* is neither pathogenic nor toxigenic and is not associated with the production of substances likely to be used as antibiotics in human or animal applications.

The culture is maintained under conditions that ensure the absence of genetic drift.

Discussion

Other than providing for an alternate source of the glutaminase enzyme preparation, there are no changes in the methods of production of Suntheanine. In the GRAS determination for the substance, it was demonstrated through HPLC and spectrographic analyses that no proteins or other foreign materials are present in Suntheanine; thus, neither fragments of the microorganism nor the enzyme are in the final product (JHeimbach 2005). Accordingly, there is no scientific rationale for further safety evaluation of the organism or the enzyme.

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Conclusion

We, the undersigned expert panel members, have individually and collectively critically evaluated the information summarized above. We conclude that L-theanine (Suntheanine®) synthesized by the enzymatic action of glutaminase derived from *Bacillus amyloliquefaciens*, a member of the *B. subtilis* group, using the same production process as Suntheanine® synthesized using glutaminase derived from *Pseudomonas nitroreducens*, shares its safety profile. The strain of *B. amyloliquefaciens* used as the source of glutaminase is fully characterized both genotypically and phenotypically. Therefore, Suntheanine® synthesized using glutaminase from *B. amyloliquefaciens* and complying with the specifications and use described in the original GRAS monograph is safe for addition to food.

It is also the Expert Panel's opinion that other scientists qualified by education and experience to evaluate the safety of food ingredients reviewing the same publicly available information would reach the same conclusion. Therefore, Suntheanine® synthesized by the enzymatic action of glutaminase derived from *B. amyloliquefaciens* is safe and is GRAS for its intended use via scientific procedures.

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SUBMISSION END

AM

Bonnette, Richard

From: Jim Heimbach [jh@jheimbach.com]
Sent: Monday, November 27, 2006 2:34 PM
To: Bonnette, Richard
Cc: Valerio, Luis Jr.
Subject: Re: question regarding L-theanine GRAS notification
Attachments: Draft Taiyo MS.pdf; 78-Week Tox & Carcinogenicity Study.pdf

Dear Richard and Luis--

I hope you both had a happy Thanksgiving!

Thank you for sending your questions via e-mail. I referred them to Joe Borzelleca and Bill Hall, the two toxicologists involved with the GRAS determination.

Their response to your first question was as follows:
In answer to your first question [regarding statistical analysis of the data in Table 1 of Borzelleca et al. 2006], statistics were not done on the kidney based on the additional evidence seen in the study that the three rats were genetically related, tumors and hyperplastic tubular lesions were multiple and bilateral in these three animals, and similar changes were not seen in any other animals of the study, a finding not consistent with chemical carcinogenesis.

Further, the microscopic appearance of the kidney tubules of the animals that had hyperplastic and neoplastic lesions was totally different from the remainder of the high dose animals (and all others) in that proliferative and degenerative changes were not seen in the latter. This finding is not consistent with treatment-related carcinogenesis. Thus, the incidence of dosed animals (females only) with tumors and hyperplastic lesions of the kidney was 3/60 while normal was 57/60 (3 dose groups of 20 each). Even if this were statistically significant, it would not be biologically significant because of these findings and therefore, indicative of another mechanism of formation.

In answer to your second question, about the Hall et al. manuscript, Bill provided the following information:
The manuscript has been accepted by Tox Path and is due to be published in Volume 35, Issue 2 in February, 2007. According to the editor, they will have an online version ready early in January. We have gone through the review process and should receive the galleys within the next two weeks. There should be few changes to the final manuscript as submitted. I should be able to download the final version with the illustrations for your review, but I will need to check with the Journal first.

Bill did send me a copy of the MS which represents the final form, after all revisions in response to editorial or reviewer comments. This is attached as "Draft Taiyo MS."

You also requested a copy of the unpublished 78-day toxicity and carcinogenicity study cited in the GRAS notice. This also is attached to this e-mail.

Please call me with any further questions regarding the GRAS determination for L-theanine.

Regards,
Jim Heimbach

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----- Original Message -----
From: Bonnette, Richard
To: jh@jheimbach.com
Cc: Valerio, Luis Jr.
Sent: Tuesday, November 21, 2006 4:20 PM
Subject: question regarding L-theanine GRAS notification

Dear Dr. Heimbach,

As discussed on the phone this morning, we've put our questions into an email for you. Let me know if we can elaborate on any of these questions.

Our first question is regarding the article by Borzelleca J, et al. (2006. Food Chem. Tox. 44:1158-1166) that describes a 13-week rodent feeding study cited by your notification in support of the safe use of theanine. In table 1 (page 1162) of the published article, the incidences of renal pathologies are reported. Are the incidences of tubular cell adenoma and tubular epithelium hyperplasia data in female rats in groups 3 and 4 statistically significant? In addition, please indicate where else statistical significance was determined for the data in table 1.

The renal histopathology findings from the above study are discussed in the notice on pages 41-42. As a result of these renal pathologies it is indicated in the notice that another investigation was undertaken and is currently in press for publication in the journal Toxicologic Pathology. (Hall et al 2006). Is this article is available to us to review in consideration of the notice? A medline search this morning for the article did not indicate that it is yet available.

A 78-week repeated dose toxicity testing of L-theanine is cited in the notice as an unpublished corroborative study. Is the report of this study is available for us to review? The exact citation you provide in the notice is as follows. *Fujii, Arihiro, Takeshima, Kaneko, and Iuchi. 1999. Repeated dose toxicity study of L-theanine by 78-week oral administration in mice.*

Thanks for your attention to these questions.

With best regards,
Richard Bonnette

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