Center for Regulatory Services, Inc.

5200 Wolf Run Shoals Road
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Smedley@cfr-services.com

Since 1973
consultants to the regulated industry

March 16, 2022

David Edwards Director
Division of Animal Feeds (HFV- 220)
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Pl.
Rockville, MD 20855

Subject: Filing of Animal GRAS Notice
Alpha-Lipoic Acid In Dry Foods for Dogs
Notifier: Hill's Pet Nutrition Inc.
400 SW 8th Avenue
Topeka, Kansas 66603

Dear Dr. Edwards:

On behalf of Hill's Pet Nutrition Inc. I am providing an Animal Generally Recognized as Safe Notice (AGRN) for the use of Alpha-Lipoic Acid in dry foods for adult (non-pregnant, non-lactating) dogs as a nutritive antioxidant. The submission is compliant with 21CFR 570.210-255. The GRAS conclusion is based on scientific procedures. We note that there has been a change in the physiological status of the intended classes of dogs from the AGRN 3 submission.

Should you have any questions on the filing, please contact me directly.

Sincerely,

Kristi Smedley

Digitally signed by Kristi Smedley
DN: cn=Kristi Smedley, o=Center for Regulatory Services, Inc., ou, email=smedley@cfr-services.com, c=US
Date: 2022.03.16 14:00:16 -04'00'

Kristi O. Smedley, Ph.D.
Consultant to Hill's Pet Nutrition Inc

cc: Michael Faurot, Hill's Pet Nutrition Inc

ATTACHMENTS:

Letter of Smedley Authorization to Represent
GRAS Notice Alpha-Lipoic Acid (via Email)

Hill's Pet Nutrition
PO Box 1658
Topeka, KS 66601-1658

Carlos A. González, Ph.D.
Director, Global Regulatory Affairs
+1 785.286.8711
carlos_gonzalez@hillspet.com

March 16, 2022

David Edwards
Director
Division of Animal Feeds, HFV-220
Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Place
Rockville, MD 20855

Subject: Authorization for Representation—Kristi O. Smedley, PhD
Filing of the GRAS Notice for Alpha-Lipoic Acid for Dog Food

Notifier: Hill's Pet Nutrition Inc,
400 SW 8th Avenue
Topeka, Kansas 66603

Dear Dr. Edwards:

We are authorizing Kristi Smedley to act on our behalf to represent Hill's Pet Nutrition in the matter of filing the GRAS notice and future communication for the use of Alpha-Lipoic Acid in dry foods for adult dogs as a nutritive antioxidant.

Her contact information:

Kristi Smedley, Ph.D.
Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192
703-590-7337
Fax 703-580-8637
Smedley@cfr-services.com

Please contact the undersigned with any questions.

Sincerely,

(b)(6)

Generally Recognized As Safe (GRAS) Notice For Use Of Alpha-Lipoic Acid In Dry Foods For Adult Dogs As A Nutritive Antioxidant

Hill's Pet Nutrition, Inc.

March 14, 2022

Table of Contents:

§ 570.225 Part 1 of a GRAS notice: Signed statements and certification.

1.1 In accord with 21 CFR 570. Subpart E. Hill's Pet Nutrition, Inc. (Hill's hereafter) is submitting a voluntary Generally Recognized as Safe (GRAS) notice for the use of α-lipoic acid in dry foods for adult dogs as a nutritive antioxidant.

1.2 *Name and address of your organization*

Hill's Pet Nutrition Inc,
400 SW 8th Avenue
Topeka, Kansas 66603

1.3 *Provide the name of the notified substance, using an appropriately descriptive term;*
The GRAS substance is alpha-lipoic acid (α-lipoic acid).

1.4 *Describe the intended conditions of use*
Alpha-lipoic acid is an ingredient in foods for adult (non-pregnant, non-lactating) dogs as a nutritive antioxidant at levels up to 150 ppm. As an antioxidant that is provided in the food, it has a role in physiological, biochemical, or cellular processes that inactivate free radicals or inhibit oxidative reactions to provide a beneficial physiological effect or significantly decrease the adverse effects of reactive oxygen or nitrogen species.

1.5 *Statutory basis for your conclusion of GRAS status*
The animal GRAS conclusion is filed based on scientific procedures in accordance with §570.30(a) and (b).

1.6 *State your view that the notified substance is not subject to the premarket approval requirements .*
The submitter has determined that the use of alpha-lipoic acid as used in dog foods intended for adult dogs (non-lactating, non-pregnant) as a nutritive antioxidant at levels up to 150 ppm is Generally Recognized as Safe (GRAS) based on scientific procedure and is thus exempt from the premarket approval requirement of the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 301 *et seq.*).

1.7 *State that, if we ask to see the data and information that are the basis for your conclusion of GRAS status, either during or after our evaluation of your notice, you will:*
Hills agrees to make the data and information pertaining to this submission available to the FDA. Hills agrees to both of the following procedures for making the data and information available to FDA:

(A) Upon FDA's request, Hills will allow FDA to review and copy the data and information during customary business hours at the address specified for where these data and information will be available to FDA; and

(B) Upon FDA's request, Hills will provide FDA with a complete copy of the data and information either in an electronic format that is accessible for FDA evaluation or on paper.

1.8 *State your view as to whether any of the data and information in Parts 2 through 7 of your GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552*

Hill's has placed proprietary and confidential information in the following sections and appendices:

Each section designated as [HILL'S CONFIDENTIAL] either in its entirety or in part, of the following sections is Hill's proprietary and confidential information.

Section 2.2
Section 2.2.1
Section 2.3
Section 2.4
Section 2.4.1
Table 2.3-1
Table 2.4-1
Table 2.4-2.
Appendix 1
Appendix 2

1.9 *Certify that, to the best of your knowledge, the GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to you and pertinent to the evaluation of the safety and GRAS status of the use of the substance;*

To the best of our knowledge and belief, this notice of GRAS conclusion is a complete, representative and balanced submission that includes unfavorable information, as well as favorable information, known to Hill's and pertinent to the evaluation of the safety and GRAS status of the use of alpha-lipoic acid as a nutritive antioxidant at levels up to 150 ppm in adult (non-lactating, non-gestating) dry dog foods.

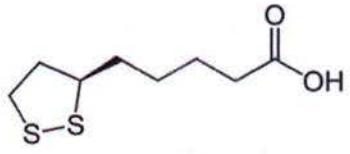
(b)(6)

Name: Michael Faurot
Title: Regulatory Affairs Manager
Hill's Pet Nutrition Inc.

§ 570.230 Part 2: Identity, method of manufacture, specifications, and physical or technical effect.

2.1 Material Characterization

Common Name:	alpha-lipoic acid (α-Lipoic acid)
CA Index Names:	dl-alpha-Lipoic acid; (RS)-1,2-dithiolane-3-pentanoic acid; (RS)-1,2-dithiolane-3-valeric acid
Other Names:	(RS)-thioctic acid; lipoic acid; α-LA; a-LA; thioctic acid; lipoate
CAS Registry Number:	1077-28-7 (dl-thioctic acid)
Other CAS Numbers:	62-46-4 (thioctic acid); 1200-22-2 (thioctic acid, d-form)
Empirical Formula:	C8H14O2S2
Molecular Weight:	206.33
Structural Formula:	Figure 2.1-1 Molecular structure of α-lipoic acid



Chemical and Physical Properties:

Appearance:	Yellow crystalline powder with a slight odor
Melting Point:	60-62 °C
Solubility:	Very slightly soluble in water, very soluble in dimethylformamide, freely soluble in methanol (Source: Eu.Ph.6.0)

Alpha-lipoic acid is a carboxylic acid consisting of a disulfide or dithiolane ring and a 5-carbon fatty acid side chain. Due to the presence of a chiral center, the alpha-lipoic acid molecule can exist in two forms, the R+ or d-form and the S- or l-form; the R-enantiomer is the naturally-occurring form and is bound to lysine of protein (Hermann et al., 1996). However, racemic mixtures of R- and S-enantiomers (dl-forms) are the most commonly used substances in alpha-lipoic acid studies, human nutritional supplements, etc. The studies that we now cite (Khanna S. et al., 1999; Zicker SC, et al., 2002; Anthony RM et al., 2021b; Wang D, et al., 2017), where the experimental animals were fed alpha-lipoic acid and an improvement in the antioxidant status was seen, were all done using a racemic mixture of R-alpha-lipoic acid and S-alpha-lipoic acid (sometimes called as d,l-alpha-lipoic acid). The material (CAS RN 1077-28-7; dl-alpha-lipoic acid) is a racemic mixture similar to those widely used in (human) dietary supplements and extensively studied; the amounts of dl-alpha-lipoic acid obtained from such dietary supplements range from 300 to 600 mg/person/day, taken in divided doses (PDR for Nutritional Supplements, 2001; Singh and Jialal, 2008). In a person weighing 60 kg, such dl-alpha-lipoic acid intakes would be equivalent to 5 to 10 mg/kg bw/day. The levels of d,l-alpha-lipoic acid that we propose for use in Hill's adult canine foods is at levels similar to those used in our studies (Zicker SC, et al., 2002; Anthony RM et al., 2021b). The data from these studies show that at these levels of d,l-alpha-lipoic acid, an improvement was seen in the intracellular antioxidant status of these dogs.

2.1.1 Naturally-Occurring alpha-Lipoic Acid

In living cells, alpha-lipoic acid is present as lipoyllysine. The structure of lipoyllysine, shown in Figure 2-2, consists of an alpha-lipoic acid moiety covalently-linked to the ε-amino group of a specific lysine residue of a target protein (Reed, 2001; Lehninger, 2005).

Figure 2.1-2 Structure of lipoyllysine

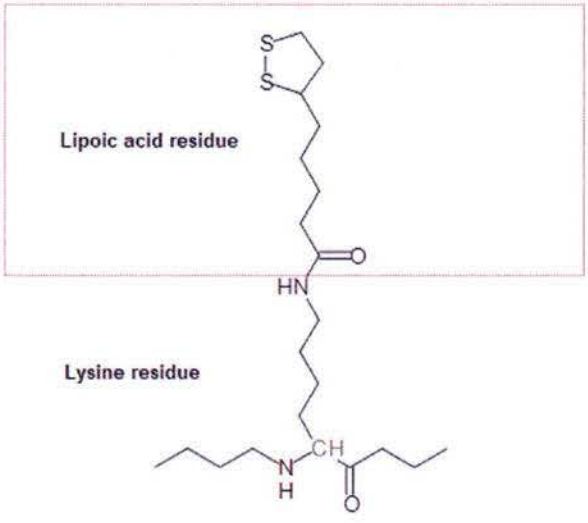


Table 2.1-1 lists amounts of alpha-lipoic acid, as lipoyllysine, naturally present in various plants, animal tissues, and some microorganisms. In plants, the highest levels are found in spinach and broccoli; in animal tissues, kidney, heart, and liver have the highest concentrations (Lodge et al., 1997).

Table 2.1-1 Sources of naturally-occurring alpha-lipoic acid

Source	Lipoyllysine Content	
	µg/g dry weight	µg/mg protein
Kidney[a]	2.64 ± 1.23	50.57 ± 5.51
Heart[a]	1.51 ± 0.75	41.42 ± 2.76
Liver[a]	0.86 ± 0.33	15.49 ± 0.01
Spleen[a]	0.36 ± 0.08	5.69 ± 1.27
Brain[a]	0.27 ± 0.08	4.85 ± 1.69
Pancreas[a]	0.12 ± 0.05	1.97 ± 0.97
Lung[a]	0.12 ± 0.08	3.20 ± 0.04
Spinach[b]	3.15 ± 1.11	92.51 ± 4.03
Broccoli[b]	0.94 ± 0.25	41.01 ± 1.02
Tomato[b]	0.56 ± 0.23	48.61 ± 1.69
Green pea[b]	0.39 ± 0.07	17.13 ± 1.23
Brussel sprouts[b]	0.39 ± 0.21	18.39 ± 2.42
Rice bran[b]	0.16 ± 0.02	4.44 ± 2.12
Yeast[c]	0.27 ± 0.05	4.49 ± 1.78
E. coli[c]	8.07	68.71 ± 11.24

Values represent mean ± standard deviation for n=4, except rice bran, n=2.
[a]Bovine acetone powders
[b]Lyophilized material
[c]Acetone powders
Method limit of detection: 0.1 µg/g dry weight

Source: Lodge et al. (1997)

Although de novo synthesis of alpha-lipoic acid in eukaryotes has not been as well-characterized as in prokaryotes (e.g., Escherichia coli), there is evidence that small amounts of alpha-lipoic

acid are synthesized in the mitochondria of plants and animals (Carreau, 1979; Reed, 2001; Zhang et al., 2003; Witkowski et al., 2007). It is presumed that alpha-lipoic acid synthesized in mitochondria is used locally, and only minor amounts are likely to enter the circulation (NTP, 2004). The mammalian lipoyllysine biosynthetic pathway is presumed to be similar to that of E. coli, illustrated in the following schematic.

Figure 2.1-3 De novo synthesis of alpha-lipoic acid in E. coli

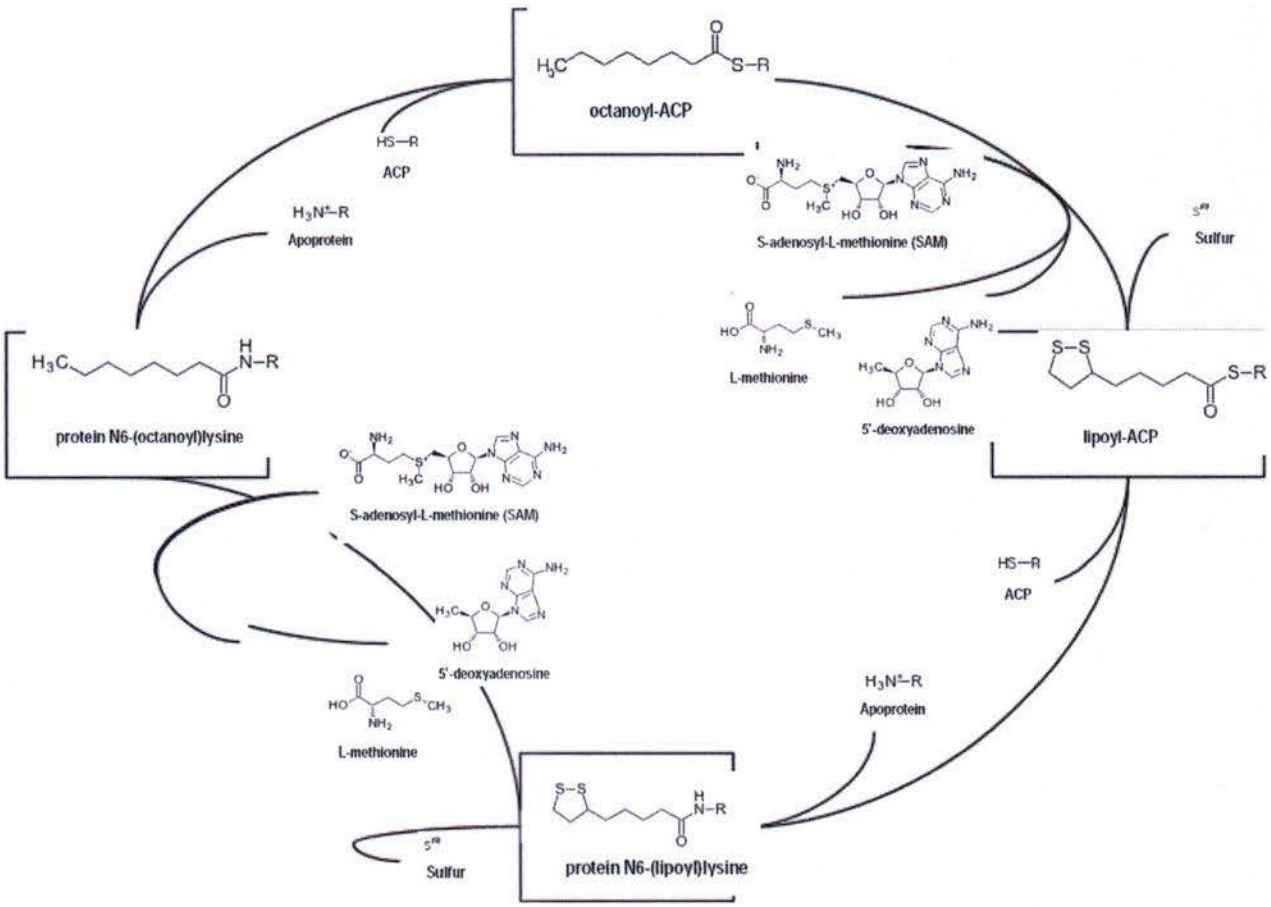
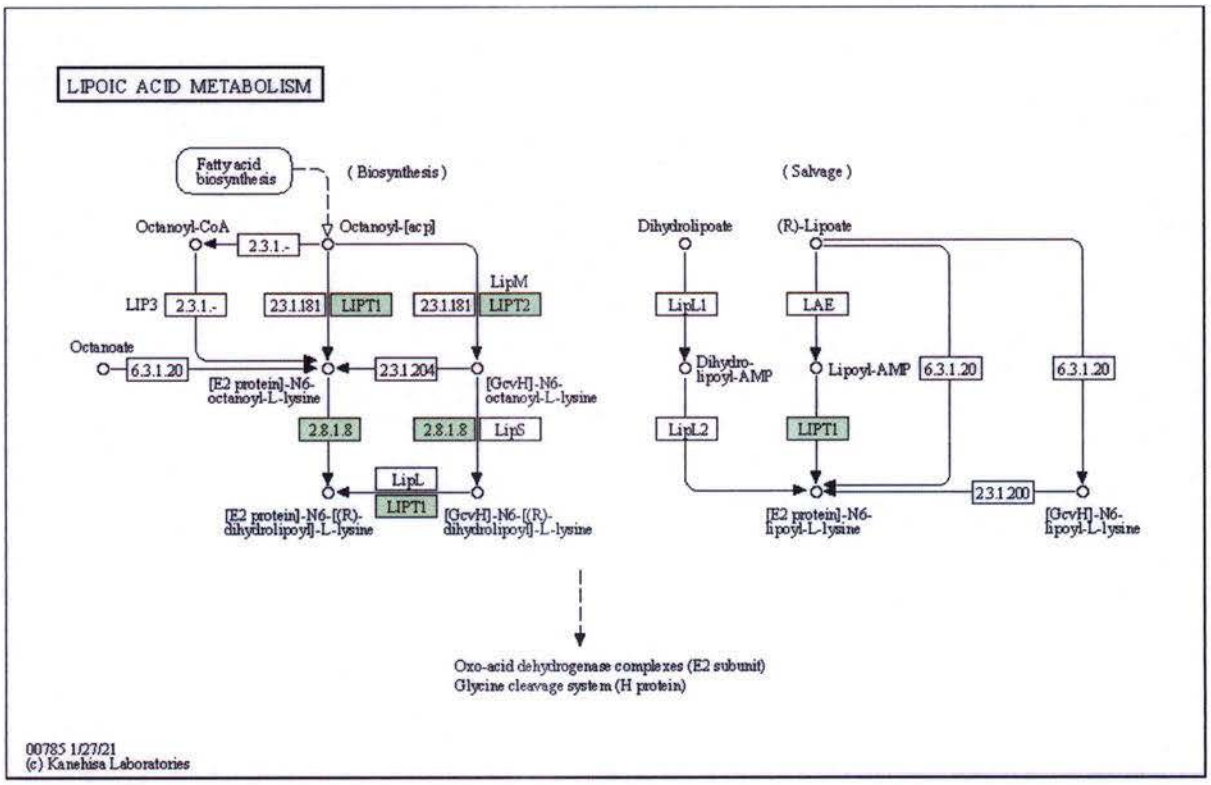


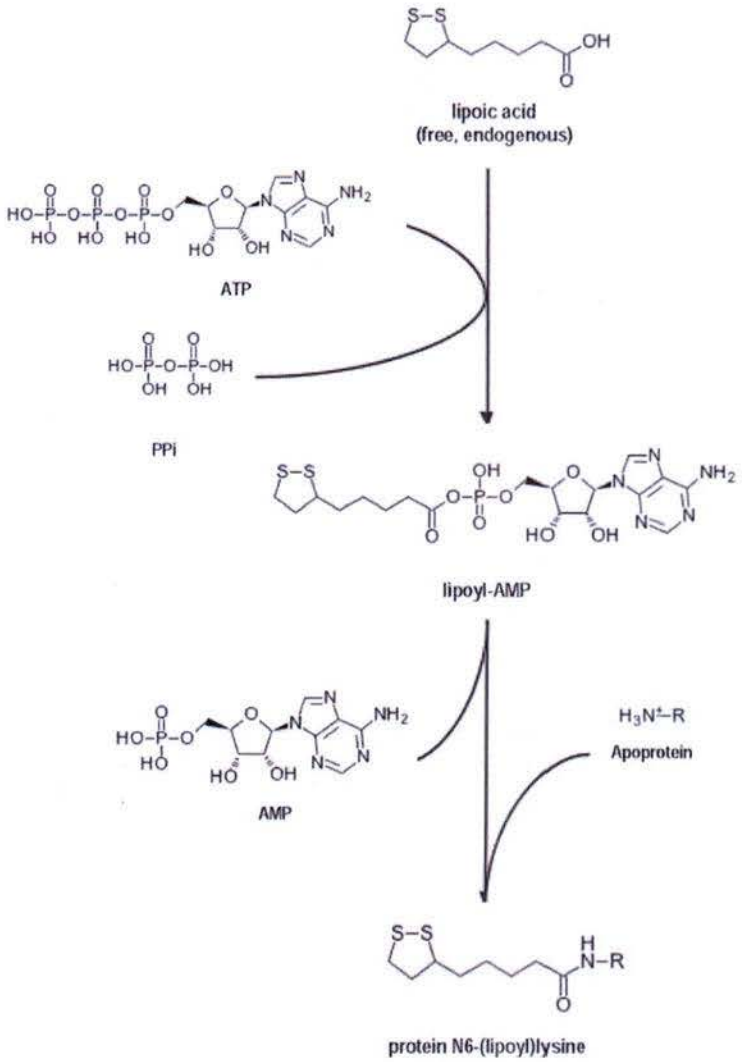
Figure 2.1-4 Lipoic Acid synthesis of alpha-lipoic acid in dogs



Source: KEGG: Kyoto Encyclopedia of Genes and Genomes. Lipoic Acid Metabolism in Canine lupis familiaris (dog), https://www.genome.jp/kegg-bin/show_pathway?cfa00785

This multistep reaction is catalyzed by a fatty (lipoic) acid synthase, which introduces two sulfur atoms at the C-6 and C-8 positions of an octanoyl moiety that is linked to a mitochondrial acyl carrier protein (ACP: malonyl-CoA) (reviewed by Witkowski et al., 2007). Octanoyl-ACP is an intermediate of fatty acid biosynthesis. Fatty acid synthesis has been shown to occur in mitochondria via a system that is distinct (type II) from cytosolic (type I) fatty acid synthesis (reviewed by Reed, 2001). The product of this process (i.e., protein N6-(lipoyllysine)) is incorporated into the appropriate mitochondrial enzyme complex. The following alternate pathway to de novo lipoate synthesis from fatty acid precursors has also been described in E. coli. This "salvage" pathway involves the conversion of endogenous free lipoic acid into lipoyllysine via a lipoyl-AMP intermediate.

Figure 2.1-5 Pathway for converting endogenous free lipoic acid to lipoyllysine
(Source: KEGG: Kyoto Encyclopedia of Genes and Genomes)



2.1.2 Manufactured alpha-Lipoic Acid

Alpha-lipoic acid is synthesized commercially through conventional processes widely used in the food industry (see section 5.0). As previously noted, racemic mixtures of R- and S-enantiomers (dl-forms) of alpha-lipoic acid are widely used in dietary supplements and have been extensively studied.

The material Hill's intends to use in canine foods (CAS RN 1077-28-7; dl-alpha-lipoic acid) is a racemic mixture produced by one or more qualified manufacturers in accordance with Good

Manufacturing Practice (GMP) standards and within the specifications established by Hill's (see section 2.3).

2.2 [HILL'S CONFIDENTIAL]: Manufacturing and Quality Assurance

[HILL'S CONFIDENTIAL]: 2.2.1 Manufacturing

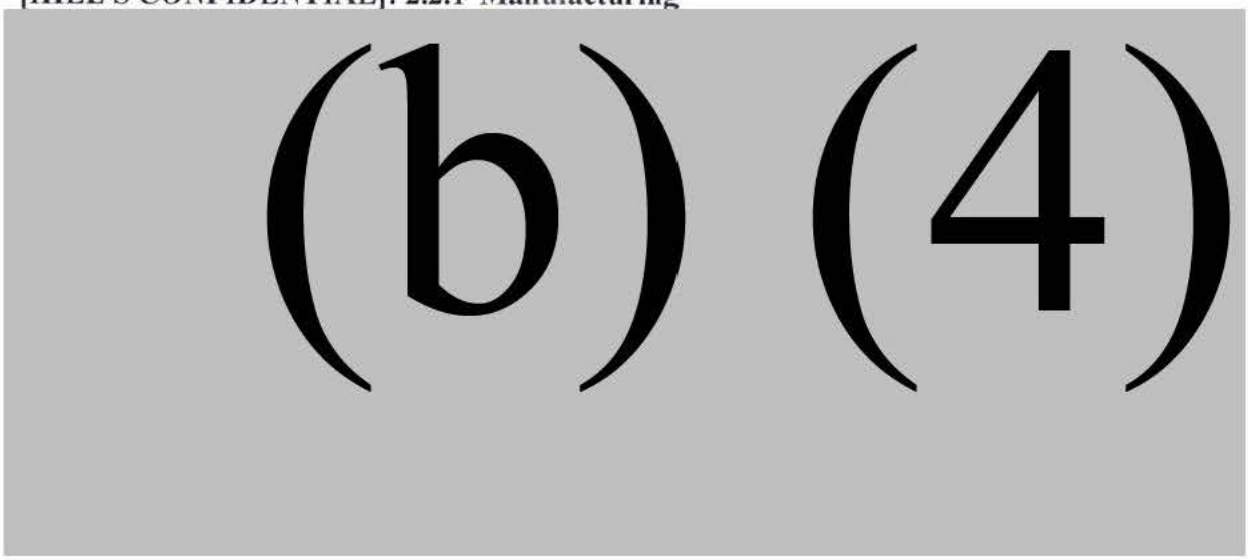
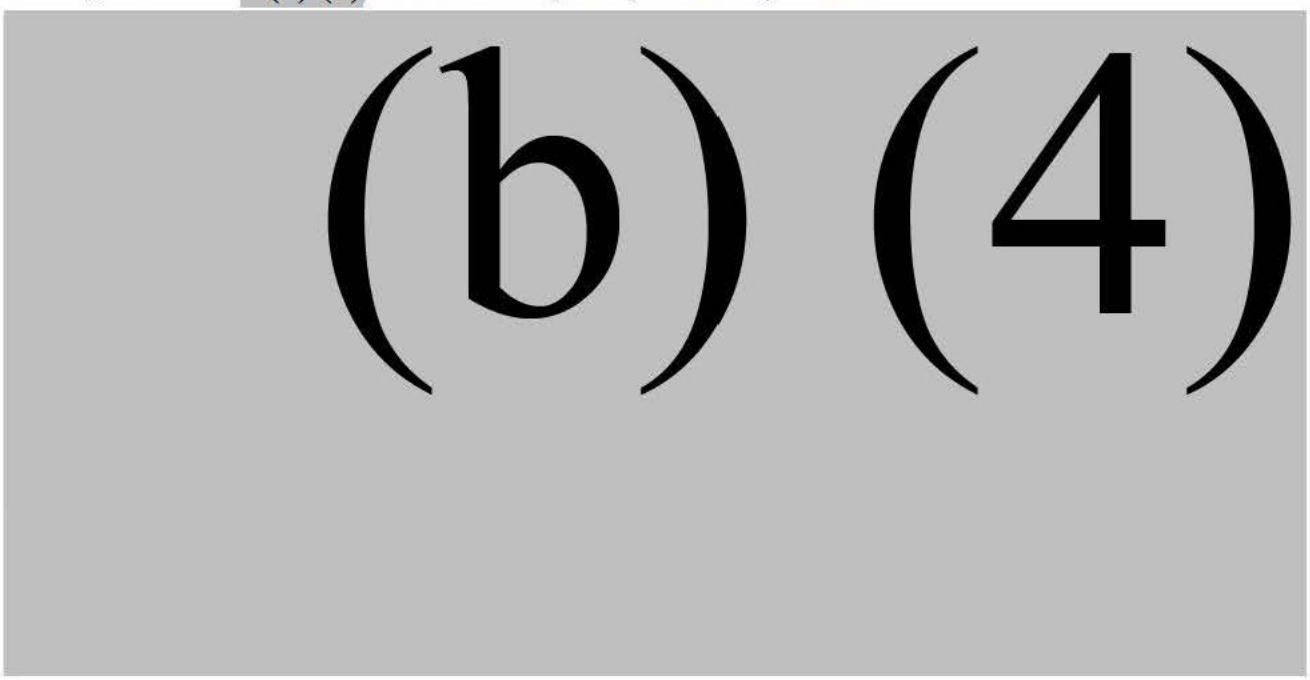


Figure 2.2 -1 (b) (4) **method for alpha-lipoic acid synthesis**



2.3 [HILL'S CONFIDENTIAL]: Specifications

(b) (4)

(b) (4)

Table 2.3-1 [HILL'S CONFIDENTIAL]: Critical elements of Hill's specifications for α-lipoic acid used in canine foods

(b) (4)

Parameter	Min.	Target	Max.	European Reference Method	US Reference Method
Parameter	Min	Target	Max	European Reference Method	US Reference Method

(b) (4)

(b) (4)

CHARACTERISTICS: TARGET AND RANGE

Parameter	Min.	Target	Max.	European Reference Method	US Reference Method
Parameter	Min	Target	Max	European Reference Method	US Reference Method
(b) (4)					

PHYSICAL CHARACTERISTICS

Grade:	n/a
(b) (4)	

PACKAGING: (b) (4)

SHELF LIFE: 1 year. As stored in a tightly-closed container in a dry, cool, and well-ventilated area, protected from light at temperatures ≤ 25 °C.

2.4 [HILL'S CONFIDENTIAL]: Quality and Stability

2.4.1 [HILL'S CONFIDENTIAL]: Alpha-Lipoic Acid

Hill's will ensure that the quality of alpha-lipoic acid used in the intended dry foods is monitored during manufacturing using validated methods.

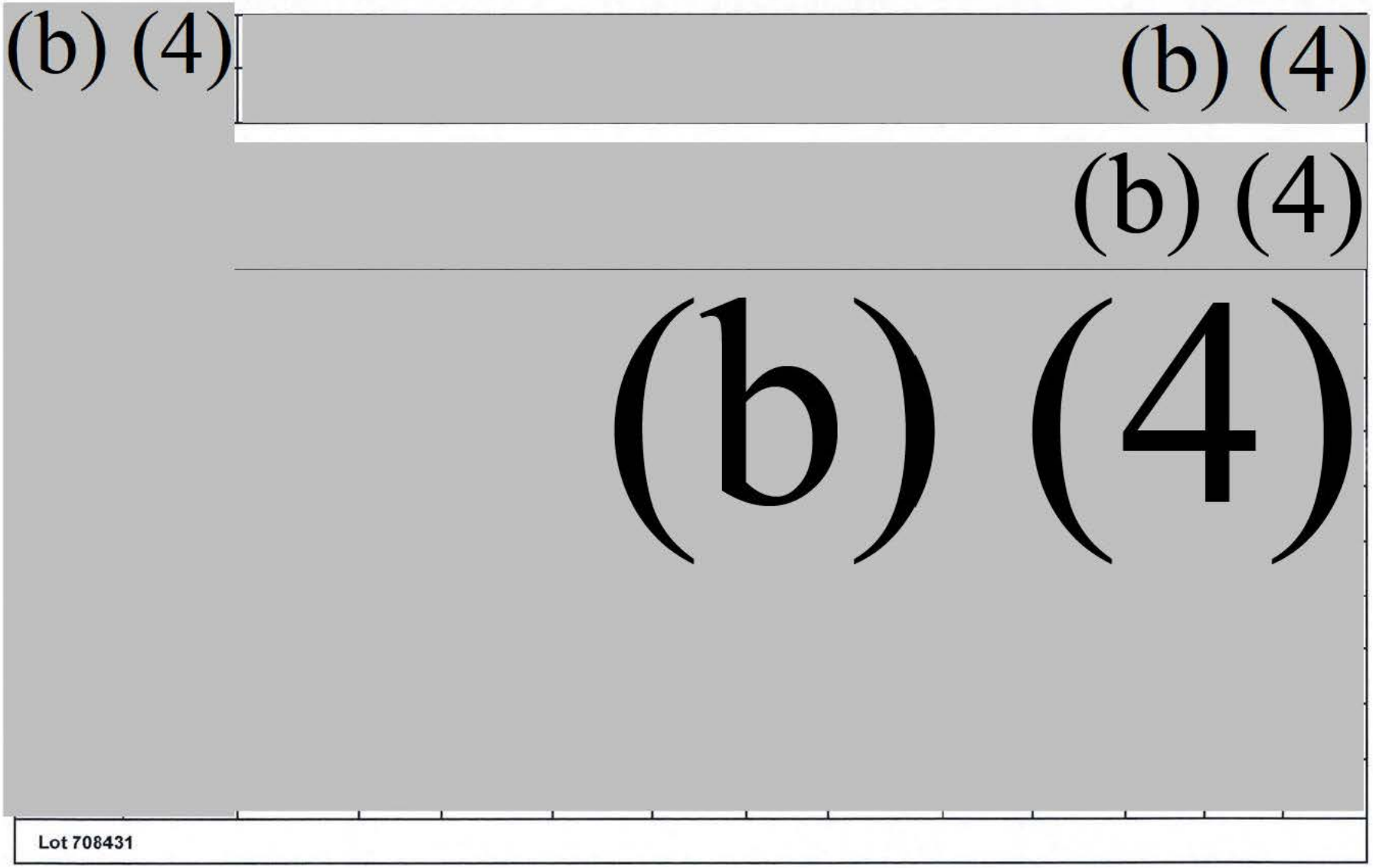
(b) (4)

Table 2.4-1 [HILL'S CONFIDENTIAL]: Results of stability testing of 3 lots of (b) (4) dl-alpha-lipoic acid stored at (b) (4) relative humidity

	Date of analysis	description
(b) (4)		

(b) (4)

(b) (4)


(b) (4)
(b) (4)
(b) (4)
(b) (4)
Lot 708431

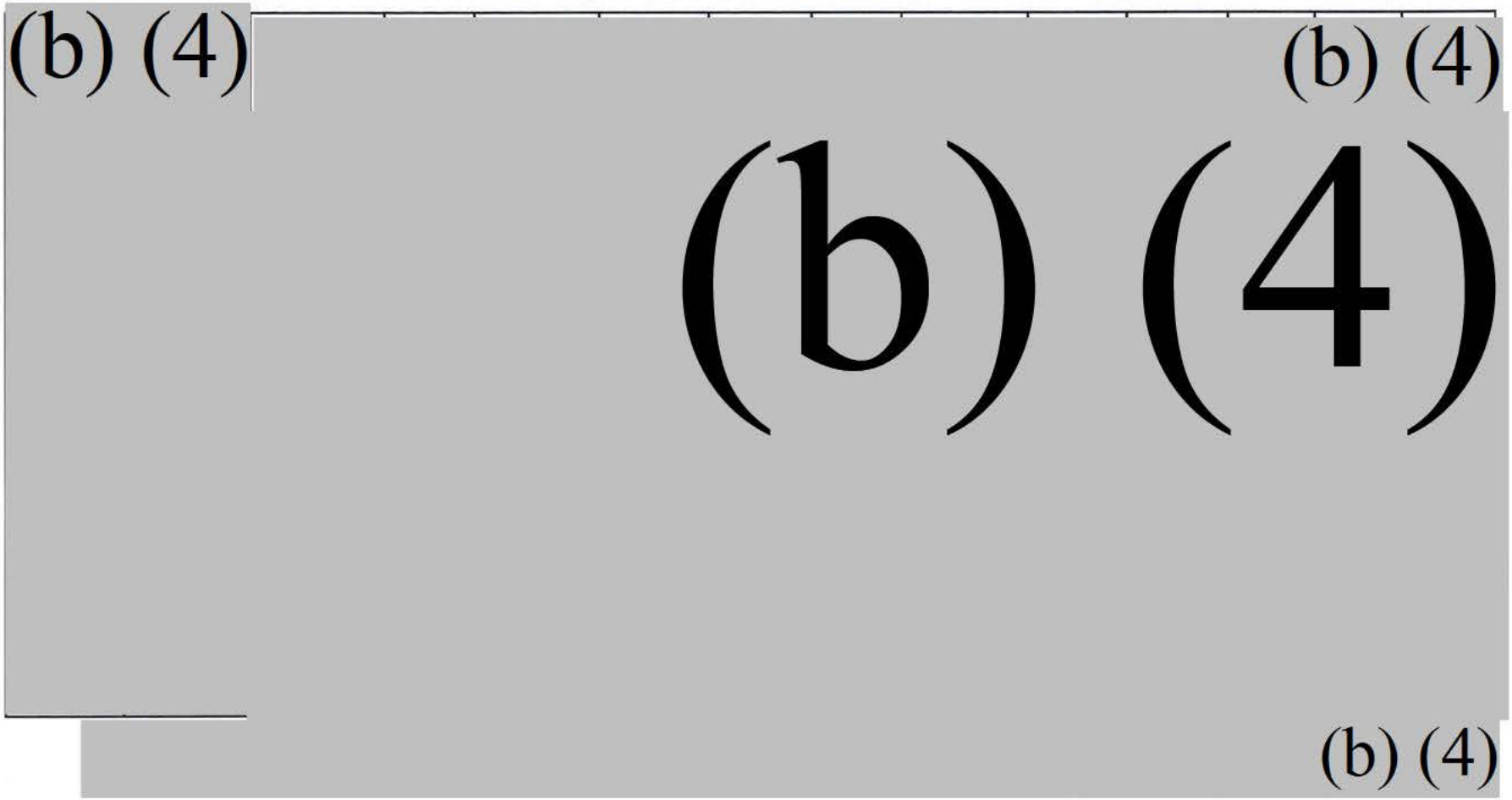

(b) (4)
(b) (4)
(b) (4)
(b) (4)

Table 2.4-2 [HILL'S CONFIDENTIAL]: Results of stability testing of 3 lots of (b) (4) *dl*-alpha-lipoic acid stored at (b) (4) relative humidity

	Date of analysis	description
(b) (4)		

(b) (4)

(b) (4)

(b) (4)

(b) (4)

(b) (4)

2.4.2 Alpha-Lipoic Acid in Canine Dry Food

Hill's has developed a method for measuring α-lipoic acid in extruded canine foods based on the published method of Witt and Rustow (1998). The method (SOP Number Version: LAB-RES-026.2), described fully in Appendix 3 (b) (4)

This method has been validated by the Hill's Science & Technology Center (Topeka, KS) and shown to be adequate for determination of lipoic acid in pet food products (see Appendix 4). The parameters characterized and the results of high performance liquid chromatography (HPLC) analysis of canine foods containing alpha-lipoic acid at various levels are provided in Appendix 5.

Appendix 6 shows data on the stability of alpha-lipoic acid in dog foods over 72 weeks. Concentrations of alpha-lipoic acid (ppm) in food with different concentrations of alpha-lipoic acid (75 ppm, 150 ppm and 300 ppm) were determined periodically over 72 weeks by HPLC. All values were within the range of expected assay sensitivity and production parameters.

2.4.3 Homogeneity

In order to determine if alpha-lipoic acid is homogeneously mixed in a canine dry food, canine dry dog food was made by adding three different levels of alpha-lipoic acid into the other ingredients and then extruded into dry kibble. Once these foods were made, five independent samples were taken from each of the three different foods and analyzed according to the method described in Witt W. and Rustow B. 1998 (See Section 2.4.2). The analyzed values of alpha-lipoic acid from the five different samples for each level of alpha-lipoic acid were averaged and the coefficient of variation (CV) was calculated. The CV standardizes the standard deviations as a function of the mean. The CVs for all the three foods were similar and very small (<10%) showing that the samples were homogeneously mixed, regardless of the levels. The results are summarized in Table 2.4-3 below.

Table 2.4-3: Results of homogeneity study when alpha-lipoic acid is added to extruded canine dry foods

Levels of a-lipoic acid	Sample	Reported Value (ppm)	Mean (ppm)	Coefficient of Variation (%)
1	1	(b) (4)	66.8 ppm	5.54
1	2			
1	3			
1	4			
1	5			
Levels of a-lipoic acid	**Sample**	**Reported Value (ppm)**	**Mean (ppm)**	**Coefficient of Variation (%)**
2	1	(b) (4)	111.8 ppm	2.23
2	2			
2	3			
2	4			
2	5			
Levels of a-lipoic acid	**Sample**	**Reported Value (ppm)**	**Mean (ppm)**	**Coefficient of Variation (%)**
3	1	(b) (4)	198.8 ppm	6.05
3	2			
3	3			
3	4			
3	5			

As a long-term established pet food manufacturer, Hill's has significant experience with manufacturing and assuring homogeneous mixing of pet foods including required minerals, vitamins, amino acids at a ppm level. Mixing procedures are frequently tested to assure the homogeneous blending of the feed ingredients.

2.5 Intended Use

(d) When necessary to demonstrate safety, relevant data and information bearing on the physical or other technical effect the notified substance is intended to produce, including the quantity of the notified substance required to produce such an effect.

Data to support intended use is only required when it is linked to safety (for example - antimicrobials, new forms of essential nutrients, products that contain fermentation media, products that increase the availability of essential nutrients, etc.). For this substance, alpha-lipoic acid, when used as a nutritive antioxidant, the use is not related to safety; for example, alpha-lipoic acid does not provide an essential nutrient nor is it intended to impact the safety of the feed. The intended use is to provide a supplemental nutritive antioxidant. A typical food for dogs includes well established antioxidants such as vitamin C, vitamin E, carotenoids, lutein, selenium, lycopene and others. Therefore, if alpha-Lipoic Acid fails as a nutritive antioxidant, it will not provide a hazard to the animal, as it is a value added portion of the diet, a supplemental nutritive antioxidant. The free radical theory (Harman D, 1972) has been widely accepted as the explanation for the basis of aging which hypothesizes free radicals as responsible for age-associated damage at the cellular level. The increasing oxidative stress in aging as a result of accumulation of free radicals is due to the imbalance between the increased free radical production and the endogenous antioxidant defenses. This imbalance with aging has been shown in multiple species (Kregel and Zhang, 2007, Pojsak, et al., 2013, and Hagen et al., 2004). Hence the interest in supplementing alpha-Lipoic Acid in dog food intended for adult dogs.

Although we understand that intended use is not needed to be demonstrated for this ingredient, Hill's has published data to support the use. A recently published systematic review of published human and dog studies (Anthony et al., 2021a) and the recently published 6-month dog study (Anthony et al., 2021b) confirm the nutritive antioxidant properties of alpha-lipoic acid. We have summarized and provided these articles to demonstrate the benefit of the use of alpha-lipoic acid in mature non-gestating and non-lactating dogs.

Summary of Anthony et al. 2021a: A systematic review of studies published from 1960 until 2021 in PubMed, Google Scholar, Cochrane Library and Medline Plus involving alpha-lipoic acid supplementation was conducted. This included a review of human clinical trials and animal studies to evaluate the utility of alpha-lipoic acid as a supplement in foods for healthy, adult dogs. An upper limit of alpha-lipoic acid intake in humans has not been conclusively established, however, the levels for oral intake of alpha-lipoic acid have been better defined in animals, and distinct species specific differences have been described. The maximum tolerated oral dose of alpha-lipoic acid in dogs has been reported as 126 mg/kg body weight (Grunert, RR, 1960) and

the LD50 as 400 to 500 mg/kg body weight (Packer L. et al., 1995). The antioxidant, anti-inflammatory and neuro-protective benefits of alpha-lipoic acid in dogs were observed at concentrations much lower than the maximum tolerated dose or proposed LD50. The review showed a study (Zicker et al., 2002) where alpha-lipoic acid increased the GSH:GSSG ratio (an important biomarker of antioxidant activity) in lymphocytes of dogs with the greatest improvement seen at 150 ppm of a-lipoic acid in the diet. Another study (Anthony et al., 2021b) showed a significant increase of intracellular glutathione in red blood cells with an increase in dietary intake of a-lipoic acid. Therefore, the review shows that at concentrations of 2.7–4.94 mg/kg body weight/day, alpha-lipoic acid is well tolerated and posed no health risks to dogs and increased the levels of the intracellular glutathione, an important marker of antioxidant capacity. The review thereby supported the use of alpha-lipoic acid as an effective nutritive additive in dog food.

Summary of Anthony et al., 2021b: A 6 month, prospective, controlled clinical trial was designed to determine the nutritive antioxidant activity of alpha-lipoic acid for adult (non-gestating and non-lactating) dogs. During a washout period of 15 months, the dogs were fed a food containing no alpha-lipoic acid to minimize the effect of confounding factors, such as Vitamin E from previous diets. Following the washout period, the dogs were randomized into four groups and fed a nutritionally complete and balanced food with either 0, 75, 150 or 300 ppm of alpha-lipoic acid for 6 months. The daily consumption of dry dog food containing up to 300 ppm alpha-lipoic acid for 6 months did not have any adverse effects on the physical appearance, body weight, food intake, serum biochemistry or hematology of healthy, adult dogs. A significant increase of 0.05ng/mL of total glutathione in red blood cell (RBC) lysate for every 1 mg/kg body weight/day increase in intake of alpha-lipoic acid was observed (see Table 2.5-1). In addition, a significant increase was observed for reduced glutathione (GSH), oxidized glutathione (GSSG) and total glutathione in RBC lysate at Month 6. The study, thereby, supported the use of alpha-lipoic acid as a safe and effective nutritive antioxidant in foods for non-gestating, non-lactating, adult dogs. Hence this study supports the feed rate of up to 150 ppm in adult dog food.

Table 2.5-1: ALA intake and glutathione levels pooled over months 2, 4 and 6 in plasma and RBC Lysates.

There was a statistically significant increase of 0.049 ng/mL of GSH and 0.047 ng/mL of total glutathione in RBC lysate for each 1 mg/kg body weight/day increase of LA intake.

		Slope		95% Confidence Interval		
Matrix	**Analyte**	**Estimate**	**SE**	**p value**	**Lower**	**Upper**
Plasma	GSH	-0.011	0.037	0.774	-0.084	0.063
Plasma	GSSG	-0.014	0.041	0.726	-0.095	0.066
Plasma	GSH/GSSG Ratio	0.005	0.031	0.880	-0.057	0.067
Plasma	Total glutathione	-0.014	0.036	0.709	-0.086	0.059
RBC Lysate	GSH	**0.049**	**0.021**	**0.024**	**0.01**	**0.092**
RBC Lysate	GSSG	0.043	0.034	0.203	-0.024	0.110
RBC Lysate	GSH/GSSG Ratio	0.006	0.029	0.846	-0.052	0.063
RBC Lysate	Total glutathione	**0.047**	**0.021**	**0.029**	**0.005**	**0.089**

Conclusion:

Supplementation of the alpha-Lipoic Acid upto 150 ppm in dog food intended for adult non-gestating, non-lactating dogs is useful as a nutritive antioxidant.

This use has been demonstrated by a systematic review of published data (Anthony et al., 2021a) and a published well controlled study assessing the various levels of alpha-lipoic acid on circulating glutathione-related compounds (Anthony et al., 2021b). However, under the regulations covering GRAS notification, the intended use is not related to safety and failure of the product would not be a hazard. As such, demonstration of the utility of the GRAS substances is not required (21 CFR 570.230(d)).

§ 570.235 Part 3 of a GRAS notice: Target animal

You must provide data and information about exposure to the target animal and to humans consuming human food derived from food-producing animals, regardless of whether your conclusion of GRAS status is through scientific procedures or through experience based on common use in food, as follows:
(a) For exposure to the target animal, you must provide:
(1) The amount of the notified substance that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet, including the intended use and all other sources in the total diet; and
(2) When applicable, the amount of any other substance that is expected to be formed in or on food because of the use of the notified substance (e.g., hydrolytic products or reaction products)
(3) When applicable, the amount of any other substance that is present with the notified substance either naturally or due to its manufacture (e.g., contaminants or by-products);

(4) The data and information you rely on to establish the amount of the notified substance and the amounts of any other substance in accordance with paragraphs (a)(1) through (a)(3) of this section that different target animal species are likely to consume in the animal food (including drinking water) as part of the animal's total diet; and

3.1 Exposure through Supplementation of Dog Food

Alpha-Lipoic Acid is intended to be added to the complete feed of non-gestating, non-lactating adult dogs at levels of up to 150 ppm (equivalent to up to 150 mg in one kg of dry dog food).

Table 3.1-1 Projected alpha-lipoic acid intakes among dogs based on normal food consumption estimates

kg	lb		
Body wt in kg	**Body wt in lb**	**Food Intake g/day**	**a-Lipoic acid intake mg/kg bw/day**
2.3	5	59	3.8
4.5	10	99	3.3
9.1	20	167	2.8
18.1	40	281	2.3
27.2	60	381	2.1
36.3	80	473	2.0
45.4	100	559	1.8

Based on inclusion of alpha-lipoic acid at 150 ppm in canine food (150 mg/kg food or approximately 42 µg/kcal). alpha-lipoic acid intake calculated using the following equation: [food intake (g/day) ÷ body weight (kg)] x 0.150. To determine µg alpha-lipoic acid/kcal, divide alpha-lipoic acid intake (in mg/kg/day) by the caloric intake (per kg bw/day) x 1000. Calculate the alpha-lipoic acid intake/kg bw as described. Then calculate caloric intake/kg bw from food intake (g)/day divided by bw (kg) x 3.53 (kcal/g diet).

Although some other typical components of dog food (see table 2.1-1) may have very low ppm levels of naturally occurring alpha-lipoic acid, at maximum, the level of alpha-lipoic acid, would be less than 155 ppm, and that would be a diet that was very high in kidneys (very unlikely).

The alpha-lipoic acid is 99.7% pure, at the very low level of incorporation in the diet (150 ppm) and the very low levels of impurities, this should not cause a concern.

3.2 Intake, Metabolism, and Elimination of Exogenous alpha-lipoic acid

Schupke *et al.* (2001) analyzed the metabolism of *dl*-alpha-lipoic acid in mice, rats, and dogs. ^{14}C-Radiolabeled *dl*-alpha-lipoic acid was administered to male NMRI mice and male Wistar rats as a single oral (gavage) dose of 30 mg/kg bw; beagle dogs received a single dose of 10 mg/kg bw by gastric lavage and, after a 7-week washout period, intravenously. Samples of plasma, urine, and feces were obtained and analyzed for radioactivity followed by HPLC.

As Table 3.2-1 shows, administration of *dl*-alpha-lipoic acid as a single oral dose to mice, rats, and dogs resulted in rapid excretion of radioactivity in the urine; more than half of the dose was excreted during the first 24 hours, suggesting extensive first-pass metabolism. Alpha-Lipoic acid was not detected in the urine of any of the species tested; it was, however, the major fraction in fecal samples, 14, 17, and 11% of the dose administered to mice, rats, and dogs, respectively. The results of plasma radiolabel analyses are summarized in Table 3.2-2.

Table 3.2-1 Mean radiolabel in urine (0-24 hours) of the mouse, rat, and dog following oral (gavage) administration of [^{14}C]α-lipoic acid as a single dose

Species	Oral dose (mg/kg bw)	Number of animals (males)	Total dose excreted (%)	Dose per metabolite fraction identified (%)								Sum of others
				M1	M2	M3	M4	M5	M7	M9	M10	
Mouse	30	15	54.7 ± 12.7	2.9	1.3	1.0	-	4.8	5.2	9.9	1.5	28.1
Rat	30	20	71.8 ± 9.4	2.2	1.0	2.3	5.8	12.2	-	5.8	-	42.5
Dog	10	3	63.5 ± 7.3	9.1	5.2	11.1	-	1.4	-	1.9	-	34.8

Source: Schupke *et al.*, 2001

Analysis of plasma and urine samples showed that *dl*-alpha-lipoic acid is extensively metabolized. The main metabolites of *dl*-alpha-lipoic acid are shown in Figure 3.2-1. Bisnorlipoic acid, derived from 3-keto-lipoic acid (M12), is a major product of β-oxidation of the *dl*-α-lipoic acid side chain. Bisnorlipoic acid is then metabolized to various products through further β-oxidation of the side chain, methylation of the 1,2-dithiolane moiety and subsequent oxidation, with slight differences among animal species in the predominant pathway. Dogs, for example, appear to have a more strongly pronounced ability than mice or rats to undergo sequential β-oxidation to form tetranorlipoic acid (M6) and its breakdown products (M10, M1, M2, and M3). As the data from Table 3.2-2 illustrate, in dogs, radiolabeled tetranorlipoic acid appeared at levels comparable to lipoic acid within 5 minutes after intravenous administration and was the primary product 10 minutes later. In mice, glycine conjugation of bisnorlipoic acid (M7) competes with β-oxidation.

Table 3.2-2 Mean radiolabel in plasma following administration of [^{14}C]alpha-lipoic acid to rats at 30 mg/kg bw orally (gavage) and dogs at 10 mg/kg bw orally (gavage) and intravenously (i.v.)

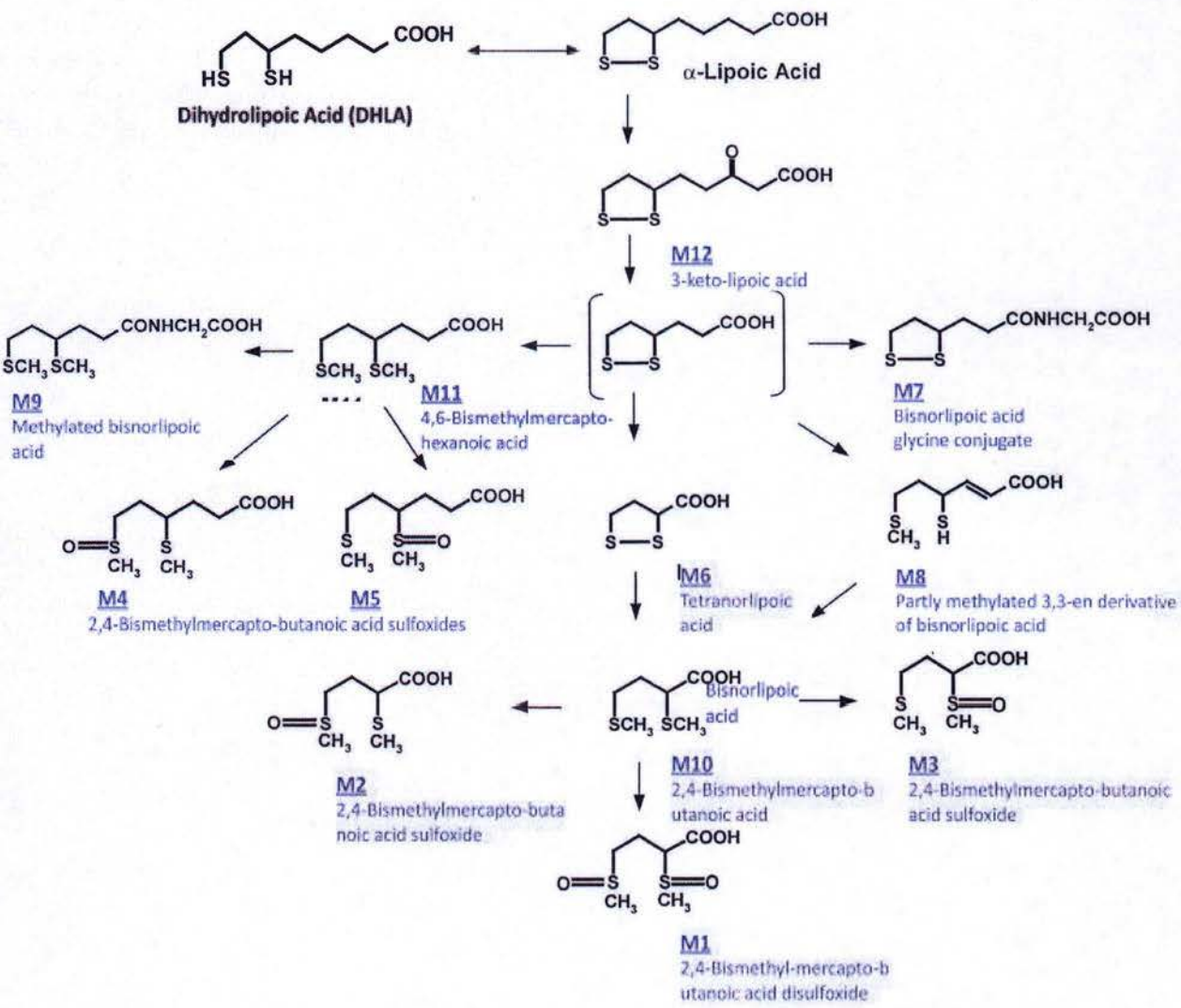
Species	Number of animals (males)	Sampling point (hours)	[^{14}C] recovery (% of dose)[a]	Relative amounts of % of radioactivity[b]							Sum of others
				M3	M6	M8	M10	M11	M12	Lipoic acid	
Rat, oral	2	1	2.9	-	-	27.0	-	29.7	10.9	-	32.4
		3	1.7	-	-	29.1	-	34.4	6.2	-	30.3
Dog, oral	3	1	4.8	-	13.9	19.3	19.7	-	-	-	47.1
		2	6.9	7.6	3.0	20.5	24.7	-	-	-	44.2
		4	3.9	20.7	-	18.1	13.8	-	-	-	47.4
Dog, i.v.	3	0.08	17.4	-	23.5	-	-	-	-	23.3	53.2
		0.25	12.2	-	44.6	-	-	-	-	-	55.4
		0.50	11.2	-	16.9	10.5	9.0	-	-	-	63.6
		1	10.2	4.3	12.8	12.6	15.4	-	-	-	54.9
		2	7.8	6.7	4.8	20.7	23.1	-	-	-	44.7
		4	4.3	8.0	-	21.9	19.8	-	-	-	50.3

[a]In the case of rats, the percentage of the dose was calculated on the basis of weight percentage of the total body weight using 4.02% for plasma, whereas for dogs the total body plasma was calculated from total blood volume (84.5 mL/kg), along with animal weights and plasma/blood ratios.

[b]Values represent relative peak areas expressed as a percentage (*i.e.*, 100% equals the sum of all peak areas in the respective radiochromatogram). The ^{14}C recoveries obtained by SPE ranged between 40 and 70%.

Source: Schupke *et al.*, 2001.

Figure 3.2-1 Overview of the main metabolites of (*dl*-)α-lipoic acid



Metabolite	Dog	Mouse	Rat	Human

	Plasma (oral/i.v.)	Urine (oral)	Feces (oral)	Plasma	Urine	Feces	Plasma	Urine	Feces	Plasma	Urine	Feces
α-Lipoic acid			√			√			√	√	√	
M1		√			√			√				
M2		√			√			√			√	
M3	√	√			√			√		√	√	
M4								√			√	
M5		√			√			√			√	
M6	√									√		
M7					√							
M8	√						√			√	√	
M9		√			√			√				
M10	√				√					√	√	
M11							√			√	√	
M12			√				√			√		

Adapted from Schupke *et al.*, 2001

Schupke *et al.* (2001) also examined metabolism in humans receiving a single dose of 600 mg *dl*-alpha-lipoic acid as an oral solution or as tablets (600 mg 3 times daily over 3 days) using LC/MS/MS. Since ^{14}C radiolabel was not administered to humans, a direct comparison to the other animal species examined was not possible. Nevertheless, the human metabolic profile showed greater similarity to lipoic acid metabolism in rodents. There was no equivalent in humans of the tetranorlipoic acid derivatives that predominate in dogs, and 3-keto lipoic acid, an intermediate in the course of β-oxidation of lipoic acid, was found in human and rat, but not dog, plasma.

Despite the slight differences in metabolism among animal species, the available data from studies with radiolabeled *dl*-alpha-lipoic acid indicate that: (1) all metabolites found within 24 hours in the urine of dogs receiving a single oral (gavage) dose of 30 mg/kg bw were also identified in the urine of mice and rats (Table 3.2-1); and (2) metabolism to tetranorlipoic acid (M6) and its derivatives occurs more rapidly in the dog, but its products were also evident in the urine of mice and rats after 24 hours.

A 2010 study (Zicker et al, 2010) of orally administered alpha-lipoic acid in dogs showed that the pharmacokinetic parameters of dl-alpha-lipoic acid were influenced by dose and the route of administration . Absorption of alpha-lipoic acid is reduced when it is used as an ingredient in extruded dog food compared to its absorption of a comparable dose of orally administered alpha-lipoic acid in the form of a capsule, given with or without food. The concentrations of dl-α-lipoic acid in plasma increased in proportion with the dose, regardless of its administration

orally in a capsule form or as an ingredient in dog food. The maximum serum concentrations and the time to reach peak concentrations of orally administered alpha-lipoic acid in plasma are within the range of values reported for other species (Hermann R. et al 1998, Teichert J., 1998, Breithaupt-Grogler, K et al 1999). The peak concentration of alpha-lipoic acid in plasma is reduced and the time to reach the peak concentration is delayed when alpha-lipoic acid is present in extruded dog food compared to the peak concentration and the time to reach peak concentration if alpha-lipoic acid is administered orally with or without food, followed by withholding of food for 12 h (Zicker et al, 2010). Delays in reaching peak concentrations in plasma in dogs fed the extruded foods may be attributable to the complex matrix used to formulate the foods and hence the slower absorption from the gastrointestinal tract.

3.3 Conclusion

Studies on intake, metabolism and elimination in mice, rats and dogs show extensive first-pass metabolism in plasma and urine, irrespective of species. No alpha-lipoic acid or metabolites in urine but major fractions were detected in feces. Pharmacokinetic study of dl-alpha-lipoic acid show that the maximum serum concentrations and the time to reach peak concentrations of dl-alpha-lipoic acid administered orally are within the ranges reported for other species. We conclude that the metabolism of dl-alpha-lipoic acid across species is comparable.

§570.240 Part 4 of a GRAS notice: Self-limiting levels of use

In circumstances where the amount of the notified substance that can be added to animal food is limited because animal food containing levels of the notified substance above a particular level would become unpalatable or technologically impractical, in Part 4 of your GRAS notice you must include data and information on such self-limiting levels of use.

There are no known self –limiting levels of use for a-lipoic acid.

§570.245 Part 5 of a GRAS notice

Experience based on common use in food before 1958.
If the statutory basis for your conclusion of GRAS status is through experience based on common use in animal food, in Part 5 of your GRAS notice you must include evidence of a substantial history of consumption of the notified substance for food use by a significant number of animals of the species to which the substance is intended to be fed prior to January 1, 1958, and evidence of a substantial history of consumption by humans consuming human foods derived from food-producing animals prior to January 1, 1958.

The animal GRAS conclusion is filed based on scientific procedures in accordance with § 570.30(a) and (b).

§ 570.250 Part 6 of a GRAS notice: Narrative

You must include a narrative that provides the basis for your conclusion of GRAS status, in which:
(a)(1) You must explain why the data and information in your notice provide a basis for your view that the notified substance is safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food-producing animals. In your explanation, you must address the safety of the notified substance, considering all animal food (including drinking water) as part of the animal's total diet, taking into account any chemically or pharmacologically related substances in such a diet. In your explanation, you must also address the safety of the notified substance in regard to human exposure, considering all dietary sources and taking into account any chemically or pharmacologically related substances;
(2) In your explanation, you must identify what specific data and information that you discuss in accordance with paragraph (a)(1) of this section are generally available, and what specific data and information that you discuss in accordance with paragraph (a)(1) of this section are not generally available, by providing citations to the list of data and information that you include in Part 7 of your GRAS notice in accordance with § 570.255;
(b) You must explain how the generally available data and information that you rely on to establish safety in accordance with paragraph (a) of this section provide a basis for your conclusion that the notified substance is generally recognized, among qualified experts, to be safe under the conditions of its intended use for both the target animal and for humans consuming human food derived from food-producing animals;

6.1 Safety Studies in the Target Animal Species (Dog)

6.1.1 Long-term Feeding Study with DL-A-Lipoic Acid

Hill's conducted and published a long-term feeding study with 5 treatments levels of α-Lipoic Acid (0, 1X, 10X, 20X, and 30X the intended use level of 150 ppm) over a one year period with adult mixed breed dogs (Robinson-Pateau, et al., 2013). The daily consumption of dry dog food containing up to 4500 ppm dl-alpha-lipoic acid for a period of one year did not have any adverse effects on physical appearance, body weight, food intake, serum hematology or biochemistry of the dogs. Any adverse effects observed during the study were not attributable to the food. No clinical signs of toxicity were observed in any of the dogs during the study. Some statistically significant changes in certain blood parameters were observed, they were not clinically relevant and values were within or near normal laboratory reference ranges. An interesting observation was that the highest food consumption per kg body weight was in the group that was fed the food with the highest dl-alpha-lipoic acid but despite the increased intake, the dogs did not gain any more weight than dogs in the other groups. This could be explained by the increased energy expenditure due to the high lipoic acid content in the food (Wang et al., 2010). The authors

concluded that levels of dl-alpha-lipoic acid of up to 3000 ppm (equivalent to 52.9 mg/kg body weight/day) did not have a negative effect on the health of dogs.

6.1.2 6-Month Feeding Study with A-Lipoic Acid in adult dogs

Hill's conducted and published a 6-month controlled clinical trial with adult dogs fed 4 treatment levels of A-Lipoic Acid (0, 0.5X, 1X, and 2X of the 150 ppm intended dose) to groups of 20 dogs, after a 15-month period of a non-lipoic acid diet (Anthony et al., 2021b). Based on the feed consumption, the mean lipoic acid exposure for the different treatment groups was calculated to be 0, 1.20, 2.7 and 4.94 mg/kg body weight/day. Evaluations included physical examinations to assess overall health of animals, hematology and serum biochemistry profiles (months 0 and 6), bodyweight (every other week), food intake (daily), and measurements of reduced glutathione, oxidized glutathione levels and total glutathione levels as well as the ratio of reduced glutathione to oxidized glutathione in both plasma and erythrocyte lysates of all animals (months 0, 2, 4 and 6). Values for hematology and serum biochemistry were compared to normal canine values to help determine overall health.

The daily consumption of dry dog food containing up to 300 ppm lipoic acid for 6 months did not have any adverse effects on the physical appearance, food intake or body weight, the serum biochemistry or hematology of the dogs. Of the adverse events observed in the study, none of them was attributed to the food. The GI complications observed in some animals were not due to a-lipoic acid supplementation in food because all cases resolved while the dogs continued to remain on the food. Sporadic health events were not related to the study, including one death due to anaplastic sarcoma. The dogs did not show any signs of clinical toxicity during the course of the study. Serum biochemistry and hematology were monitored at the beginning of the baseline period and at the beginning and end of the alpha-lipoic acid feeding period of the study and were interpreted not to be clinically significant. The values of all blood parameters stayed within normal laboratory reference ranges.

The study demonstrated that the inclusion of dl-α-lipoic acid of up to 300 ppm (equivalent to 4.94 mg/kg body weight/day) posed no health risks and thereby supports its use as a safe nutritive antioxidant for non-gestating, non-lactating adult dogs.

6.1.3 Safety Conclusion based on Target Species Studies

Previous studies with dl-alpha-lipoic acid show that the maximum tolerated dose of alpha-lipoic acid in dogs was estimated to be 126 mg/kg body weight (Grunert, RR, 1960) and the proposed LD50 of alpha-lipoic acid in dogs administered orally was reported to be between 400 and 500 mg/kg body weight (Packer L. et al. 1995). In studies published by Hill's on alpha-lipoic acid oral supplementation, dogs were fed between 0 and 87.7 mg/kg body weight per day (0-4.94 mg/kg body weight per day in Anthony et al, 2021b, 2.5-25 mg/kg body weight/day in Zicker et al, 2010, 0-85 mg/kg body weight per day in Zicker et al, 2002 and 0.31-87.7 mg/kg body weight per day in Pateau-Robinson et al, 2013). The inclusion of alpha-lipoic acid at these concentrations did not pose any health risks to the animals. Hill's intends to use a-lipoic acid in dry foods for adult dogs at levels up to 150 ppm (150 mg/kg food or 0.0150%). At these levels, the average lipoic acid exposure in dogs is calculated to be approximately 2.7 mg/kg body weight of the dog/day. This inclusion rate is much lower than the maximum tolerated dose of 126 mg/kg body weight/day and the oral LD50 is 400–500 mg/kg body weight/day. Other published articles on studies of oral alpha-lipoic acid supplementation in canines also did not report adverse events or any health risk to the dogs at the concentrations of inclusion (2 mg/kg body weight/day in Williams DL. 2017, 11 mg/kg body weight/day in Milgram NW et al, 2007). These studies support the use of dl-alpha-lipoic acid as a safe nutritive antioxidant in food for non-gestating, non-lactating adult dogs.

6.2 Safety Studies in Other Animal Species

A number of studies were found in published scientific literature that examined the oral toxicity of alpha-lipoic acid in other animal species. The results of these studies show that single oral (gavage) doses up to 2000 mg/kg bw of *dl*-alpha-lipoic acid are not lethal to rats (Cremer *et al*, 2006a). The no-observable-adverse-effect level (NOAEL) in rats following oral exposure *via* gavage for 4 weeks or in the diet for up to 2 years was approximately 60 mg/kg bw/day. These studies are discussed in more detail in subsequent sections and are summarized in Table 6.3-1.

6.2.1 Single-Dose Toxicity in Rodents

According to Fuke *et al.* (1972), the oral median lethal dose (LD_{50}) of alpha-lipoic acid in male and female 7-week-old Sprague-Dawley rats was 1320 and 1130 mg/kg, respectively; the maximum non-lethal oral dose was 500 mg/kg for males and 350 mg/kg for females.

Cremer *et al.* (2006a) examined the acute toxicity of alpha-lipoic acid in 8-week-old female Sprague-Dawley IGS BR rats using the up-and down-procedure described in OECD[1] Test Guideline 425 (2001). A single dose of 175 mg/kg bw (in 0.1% aqueous solution of sodium carboxymethyl cellulose) was administered to 1 rat, followed by 550 mg/kg bw in a second rat, and ultimately 2000 mg/kg bw in 3 rats. Animals were observed for mortality and other signs of toxicity at regular intervals during the first 8 hours and for 14 days after dosing. Body weights were recorded prior to dosing and on Days 7 and 14. No mortality or signs of toxicity were noted

[1] OECD: Organization for Economic Cooperation and Development.

at 175 or 550 mg/kg bw. Animals receiving 2000 mg/kg bw exhibited sedation, apathy, piloerection, hunched posture and/or eye closure between 2 and 6 hours after dosing, but no mortality. No other effects were noted. The oral median lethal dose (LD_{50}) of α-lipoic acid in this study was considered to be higher than the highest dose administered (>2000 mg/kg bw).

6.2.2 Four-Week Oral Toxicity in SD Rats (Cremer *et al.*, 2006a)

6.2.2.1 Study Design

Following a dose-range finding study (68.1, 147, 316, or 681 mg/kg bw/day alpha-lipoic acid given to Wistar rats *via* oral gavage for 2 weeks), Cremer *et al.* (2006a) administered alpha-lipoic acid *via* oral gavage to Wistar (Hsd/Win:WU) rats (5/sex/group for toxicokinetics and 10/sex/group for all other evaluations) at 0 (1,2 propylene glycol vehicle), 31.6, 61.9, or 121 mg/kg bw/day for 4 weeks. Animals were monitored for mortality twice per day, and food consumption, body weights, reflexes, behavior, and general condition were evaluated weekly. Ophthalmic (control and high-dose groups only), hearing, and dental examinations were conducted prior to dosing and during test week 4. Hematology (erythrocytes, hematocrit, hemoglobin, leukocytes) and clinical chemistry (alanine aminotransferase, albumin, alkaline phosphatase, aspartate aminotransferase, urea, calcium, chloride, creatine kinase, creatinine, ©-glutamyltransferase, glucose, glutamate dehydrogenase, inorganic phosphate, potassium, sodium, total bilirubin, total cholesterol, total protein, and triglycerides) parameters were evaluated during Weeks 1 and 4. At the end of the study, animals underwent a full necropsy. The weights of the adrenals, brain, female genital tract, heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes, and thymus were recorded. Several tissues from the control and high-dose groups, and the liver, kidneys, lungs, and mammary glands of animals in the low- and mid-dose groups, were preserved and examined microscopically.

6.2.2.2 Mortality, Clinical Signs, Body Weights, etc.

No deaths occurred in any group. The low- and mid-dose groups exhibited no test article-related effects. Clinical symptoms such as reddish incrustations of the nose or eyes, eschar formation, wounds, or focal alopecia on different locations were present in animals of both sexes and in all study groups, including controls. These findings were therefore considered incidental. At 121 mg/kg bw/day, alpha-lipoic acid produced slight hypokinesia in 1 male for 3 days during Week 4. These symptoms were first observed between 45 and 180 minutes after dosing and lasted for a day. Several females in this group exhibited coordination disturbances (staggered and stilted gait) within 30 to 180 minutes after dosing. One female showed reddish salivation and another had slight clonic convulsions on a single occasion. There was no evidence of treatment-related adverse effects on body weight, feed consumption, reflexes, hearing, dentition status, ophthalmological assessments, or urinalysis.

6.2.2.3 Clinical Chemistry and Hematology

Slightly, but significantly, lower red blood cells, hematocrit, and platelets counts were observed in females receiving 61.9 mg/kg bw/day at Week 4, compared to the control group. No effects on hematological parameters were observed in any other group. The lack of a dose-response and the occurrence in one sex would therefore suggest these variations were incidental and not treatment-related.

There were no statistically significant differences in clinical chemistry measures in the 31.6 and 61.9 mg/kg bw/day groups compared to control. Male rats in the 121 mg/kg bw/day group had significantly lower cholesterol that persisted until Week 4. Lower total protein and triglyceride levels, and slightly higher alanine aminotransferase and glutamate dehydrogenase levels were also noted in this group. High-dose females had slightly, but significantly, higher blood urea and cholesterol levels. Other findings, including changes in α_1- and γ-globulin, and glutamate dehydrogenase levels were observed in various groups at various time points, but all were considered to be random variations unrelated to alpha-lipoic acid treatment.

6.2.2.4 Organ Weights and Histopathology

No gross pathology findings related to treatment were found. No significant differences in absolute or relative organ weights were observed in males from the 31.6 or 61.9 mg/kg bw/day groups. However, at 121 mg/kg bw/day, males had significantly higher liver (relative) and kidney weights (absolute and relative). A statistically significant, dose-dependent increase in relative liver weights was seen in female rats. Relative kidney weights were also significantly higher among females receiving 31.6 or 61.9 mg/kg bw/day of ALA; absolute kidney weights were significantly higher in 121 mg/kg bw/day females. The effects on liver weights were considered adaptive effects, possibly associated with enzyme induction, and not indicative of hepatic toxicity; the effects on kidney weights were not accompanied by any histopathological changes and were therefore considered of no toxicological significance.

Histopathological examinations revealed some minor treatment-related effects in the liver and mammary gland; most were confined to the high-dose group. High-dose males had a higher incidence of centrilobular hypertrophy than control males (8/10 *vs.* 5/10); the severity of this lesion was also slightly greater (1.6 *vs.* 1.0). The cytoplasm of these hepatocytes was deeply eosinophilic and contained basophilic cords presumed to represent proliferated rough endoplasmic reticulum. Other effects observed with greater frequency and/or severity among alpha-lipoic acid-treated animals included rarefied periportal hepatocytes (due to lipid vacuoles) often accompanied by cytoplasmic basophilia. These changes may constitute an adaptive rather than toxic response. Centrilobular hypertrophy, for example, is typically associated with induction of phase I metabolic enzymes. The severity of hepatic microgranulomas was marginally to slightly greater among high-dose males and females compared to their control counterparts. However, the incidence of this lesion was unaffected by alpha-lipoic acid treatment. In these animals, hepatic microgranulomas tended to be larger and more frequent than in control rats. The microgranulomas consisted largely of macrophages and were frequently

associated with hepatocyte single-cell necrosis. There were, however, no differences between high-dose and control animals in the reported incidence of single-cell necrosis.

The mammary gland of high-dose group female rats also had a marginally higher incidence of diffuse hyperplasia. The mammary gland of most male rats of all treatment groups and control group showed diffuse proliferation of glandular tissue, which is a common finding in this rat strain.

The no-observable-adverse-effect level (NOAEL) in this study was considered to be 61.9 mg/kg bw/day of alpha-lipoic acid.

6.2.3 Two-Year Dietary Study in SD Rats (Cremer *et al.*, 2006b)

6.2.3.1 Study Design

Cremer *et al.* (2006b) examined the toxicity of alpha-lipoic acid in rats following administration in the diet for 2 years. Male and female Sprague-Dawley (Hsd/Win:WU) rats 38 to 42 days old (body weight of ~100 g) received diets containing 0, 20, 60, or 180 mg racemic (*dl*) alpha-lipoic acid per kg bw per day. Alpha-Lipoic acid was added to the diet daily in a solution of 1,2-propylene glycol. The amount of test substance added to the feed was adjusted on a weekly basis to compensate for body weight gains. The control and high-dose groups each consisted of 50 animals/sex/group; the remaining groups had 40 animals/sex/group. Ten rats of each sex in the high-dose and control group were killed after 1 year of treatment and underwent a complete necropsy, leaving a nominal 40 animals/sex in all groups to complete the 2-year dosing period. Mortality, food consumption, general condition and behavior, and response to stimuli were recorded daily. Body weights were measured twice weekly for 6 months and approximately monthly thereafter. Ophthalmologic and dental examinations, and a hearing function test were performed at the 1-year time point and at the end of the study. Animals from both the interim and terminal sacrifice underwent a full necropsy that included organ weights (heart, liver, lungs, spleen, kidney, adrenals, thymus, pituitary gland, gonads, thyroid, brain), bone marrow smears, and histopathology of control and high-dose group organ/tissues (heart, lungs, pleural space, liver, spleen, kidneys, adrenals, thymus, pituitary, gonads, thyroid, brain, eyes, bladder, bone marrow, trachea, aorta, esophagus, pancreas, tongue, prostate, lymph nodes, peripheral nerve, skeletal muscle) as well as any gross lesions. Clinical chemistry parameters (liver function, SGPT, creatinine, glucose, urea, SGOT, alkaline phosphatase, bilirubin, total protein, sodium, potassium, chloride, CO_2, uric acid), hematology (hemoglobin, erythrocytes, leukocytes, differential leukocytes count, hematocrit, thrombocytes, reticulocytes, and prothrombin time), and urinalysis (color, specific gravity, pH, protein, ketone bodies, glucose, hemoglobin, bilirubin, microscopic examination of sediment) were also measured.

6.2.3.2 Mortality, Clinical Signs, Body Weights, etc.

As Table 6.2-1 illustrates, mortality in the alpha-lipoic acid treatment groups tended to be lower than controls. No deaths occurred before month 15; deaths were generally preceded by a 1- to 4-week period of apathy or in some cases ataxia, loss of appetite followed by rapid weight loss,

and lack of grooming. The cause of death was determined to be pneumonia unrelated to treatment.

Table 6.2-1 Spontaneous deaths among rats receiving alpha-lipoic acid in the diet for up to 2 years

Dose (mg/kg bw/day)	N (animals/sex/group)	Males	Females
0	40[1]	10	9
20	40	10	5
60	40	3	8
180	40[1]	3	7

Deaths occurred at or after 15 months of treatment.
[1]Number of animals was 50 at study start; 40 animals remained following interim sacrifice at 12 months.

No treatment-related effects on behavior or hearing function were noted. No effects on body weight or body weight gain were observed in low- or mid-dose group. Food consumption among high-dose males and females was reduced, as were body weight gains (after at least 8 weeks of treatment) and terminal body weights (~13 % lower than control in males and 22 % lower in females). Mean body weights at study start, 1 year, and study end are summarized in Table 6.2-2.

Table 6.2-2 Mean body weights of rats receiving α-lipoic acid in the diet for up to 2 years

Sex	Dose (mg/kg bw/day)	Body weight (g)		
		Study start	12 months	24 months
Male	0	102.3 ± 1.9	506.8 ± 28.8	574.2 ± 47.9
	20	102.5 ± 1.7	511.6 ± 24.1	557.9 ± 63.6
	60	102.4 ± 2.1	506.7 ± 19.5	551.3 ± 56.8
	180	102.4 ± 1.8	469.2[a] ± 24	500.8[a] ± 56.3
Female	0	102.1 ± 1.7	306 ± 24.9	361.6 ± 35.9
	20	102.4 ± 1.4	296.7 ± 41.3	347.6 ± 48.5
	60	102.3 ± 2	304.5 ± 21.1	334.8 ± 70.5
	180	101.9 ± 1.6	265.3[a] ± 17.8	280.2[a] ± 36

[a]Students t-test, significance <0.05 in comparison to control value.

Increasing bilateral opacity of the vitreous body was observed in 1 or 2 animals in the low- and mid-dose groups between 14 and 20 months. However, the low number of animals, the absence

of a dose-response, and the known occurrence of such alteration in untreated aging rats of this strain, suggest this effect was unrelated to treatment.

6.2.3.3 Clinical Chemistry, Hematology, and Urinalysis

Clinical chemistry, hematology, and urinalysis parameters after 1 or 2 years of treatment were unaffected by treatment.

6.2.3.4 Organ Weights and Histopathology

After 1 year, there were no significant differences in organ weights (absolute or relative). After 2 years, lower absolute organ weights were observed in mid-dose (adrenal) and high-dose (heart and thymus) males, and in high-dose females (liver and lung) compared to their control counterparts. However, no significant differences were noted in organ weights relative to body weights. As Table 6.2-3 illustrates, macroscopic and histopathological examinations revealed the presence of neoplasms in both control and treated animals, with no differences in the overall incidence. The majority of neoplasms were reticuloendothelial cell sarcomas (histiocytic lymphomas), evenly distributed across treatment and control groups.

Table 6.2-3 Summary of neoplastic findings in SD rats receiving alpha-lipoic acid in the diet for up to 2 years

	Treatment group (mg/kg bw/day)							
	Males				Females			
	0	20	60	180	0	20	60	180
Tumor prevalence (% of animals with a tumor)	27.5	25	25	30	30	25	25	30
Adenoma								
Liver			2		1	1	1	2
Skin		1			2		1	
Pituitary gland	2	3	2			1	1	1
Mammary gland					2			
Thyroid gland	1	1						2
Adrenal gland						1		
Thymus	1							
Pancreas								1
Testes		1			-	-	-	-
Fibroma								
Skin					3	2		
Carcinoma								
Mammary gland					1			
Testes	1	1	1	2	-	-	-	-
Sarcoma								
Heart (NOS)				1				
Spindle cell	1							
Skin				1		1		
Parotid gland					2			
Thymus	1							1
Uterus	-	-	-	-	1			
Reticuloendothelial cell (histiocytic lymphoma)	7	7	4	8	10	6	7	9

40/sex/group

The no-observable-adverse-effect level (NOAEL) among SD rats receiving alpha-lipoic acid in the diet for up to 2 years was considered to be 60 mg/kg bw/day.

6.3 Genetic Toxicity

The potential of alpha-lipoic acid to induce mutations or chromosomal damage was evaluated by Cremer *et al.* (2006a). The results of these studies, discussed in more detail below, show no evidence of mutagenic or clastogenic potential.

6.3.1 Ames Bacterial Mutagenicity Assay (Cremer *et al.*, 2006a)

Cremer *et al.* (2006a) examined the mutagenic potential of alpha-lipoic acid using the Ames bacterial mutagenicity assay as recommended by OECD guidelines and under GLP standards. The OECD-recommended *Salmonella typhimurium* strains TA100, TA1535, TA1537, TA98, and TA102 were studied, along with TA97, which has been shown to be particularly sensitive to endogenous sulfhydryl compounds such as glutathione and L-cysteine. At levels ranging from 15.8 to 5000 µg/plate, alpha-lipoic acid was not mutagenic in TA100, TA1535, TA1537, TA98, or TA102 in the presence or absence of an S9 metabolic fraction. In the absence of the S9 mix, TA97 exhibited no mutagenicity in either the plate incorporation or pre-incubation assay. When the metabolic fraction was present, no mutagenicity was evident in this strain in the pre-incubation assay, but a possible weak effect was observed at 5000 µg/plate in the plate incorporation assay. However, by the standards of the Ames assay, the difference from the solvent controls (1.4- to 2-fold) was not substantial and alpha-lipoic aicd would therefore be considered non-mutagenic in TA97.

6.3.2 *In vivo* Mouse Micronucleus Assay (Cremer *et al.*, 2006a)

Cremer *et al.* (2006a) examined the potential of α-lipoic acid to induce chromosomal damage in mouse erythrocytes. This test was conducted in compliance with GLP standards. Hsd/Win:NMRI mice received a single oral dose of 1,2-propylene glycol (negative control, N=12 animals/sex), alpha-lipoic acid (825 mg/kg bw, N=19 males, 17 females), or cyclophosphamide (31.6 mg/kg bw, N=6 animals/sex). Animals were observed for signs of toxicity during the first 5 to 6.5 hours, followed by regular observation on Days 2 and 3. Full necropsies were conducted on animals that died during the observation period. All other surviving animals were sacrificed via CO2 inhalation after 24 hours (positive control: all mice; negative control: 6/sex; alpha-lipoic acid: at least 5/sex) or 48 hours. The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) was determined from bone marrow (femurs) smears for each sex in each group at each time point.

Ten mice (6/19 males, 4/17 females) treated with 825 mg/kg bw of alpha-lipoic acid died. Necropsy of these animals revealed no significant abnormalities. On the day of dosing, alpha-lipoic acid-treated mice exhibited slight hypokinesis (1/19 males), stilted gait (1/19), clonic convulsions (slight: 6/19 males, 5/17 females; moderate: 9/19 males, 10/17 females;

severe: 4/19 males, 2/17 females), tonic convulsions (2/19 males, 1/17 females), piloerection (1/19 males), and sunken sides (13/19 males, 3/17 females). In some cases, these effects lasted until the death of the animal. No abnormal clinical signs were observed in negative or positive control animals. The deaths were attributed to greater susceptibility among mice to the acute toxic effects of alpha-lipoic acid.

Microscopic examination of the bone marrow smears revealed a significant increase in the number of PCEs in animals receiving the positive control, as expected, but no significant differences between the alpha-lipoic acid-treated and negative control groups, suggesting alpha-lipoic acid does not induce chromosomal damage.

Table 6.3-1 Summary of toxicological assays of alpha-lipoic acid in rodents

Endpoint	Test System	Test Material	Dosage or Concentration	Result	OECD/ GLP Compliance	Reference
Acute oral (gavage) toxicity	Rat Sprague-Dawley IGS Br Female Up-and-down test method	alpha-lipoic acid (racemic), 99.0 % purity	Single dose starting with 175 mg/kg bw in 1 rat, followed by 550 mg/kg bw in a second rat, and 2000 mg/kg bw in 3 other rats; 14-day observation period.	LD_{50}: > 2000 mg/kg bw <u>175 and 550 mg/kg bw</u> No mortality or signs of toxicity. <u>2000 mg/kg bw</u> No mortality but sedation, apathy, piloerection, hunched posture, and/or eye closure noted within 2-6 hr post-dose.	OECD Test Guideline 425 GLP-compliant	Cremer *et al.* (2006a)
Acute oral toxicity	Rat Sprague-Dawley 10/sex/group	*dl*-thioctic acid	Single dose. Amount administered not specified. Procedure used for oral administration (*e.g.*, gavage) not specified.	LD_{50}: 1320 mg/kg bw in males; 1130 mg/kg bw in females Maximum non-lethal oral dose: 500 mg/kg in males; 350 mg/kg in females	Not specified	Fuke *et al.* (1972) (translation of Japanese article)
Subchronic oral (gavage) toxicity Dose-range finding	Rat Wistar	α-lipoic acid (racemic), 99.0 % purity	Doses <u>(mg/kg bw/day)</u> 68.1 147 316 681 Administered for 2 weeks.	NOAEL: 68.1 mg/kg bw/day <u>68.1 mg/kg bw/day</u> No adverse effects noted. <u>147 mg/kg bw/day</u> Severe symptoms of toxicity (hypokinesia, coordination disturbances, sunken sides, and clonic convulsions) noted. <u>316 and 681 mg/kg bw/day</u> Lethal effects.	Not specified	Cremer *et al.* (2006a)

Table 6.3-1 Summary of toxicological assays of alpha-lipoic acid in rodents (Cont'd)

Endpoint	Test System	Test Material	Dosage or Concentration	Result	OECD/ GLP Compliance	Reference
Subchronic oral (gavage) toxicity	Rat Wistar 15/sex/group	alpha-lipoic acid (racemic), 99.0 % purity	Doses (mg/kg bw/day) 0* 31.6 61.9 121 Administered for 4 weeks. *Vehicle: 1,2-propylene glycol	NOAEL: 61.9 mg/kg bw/day No mortality in any group. Statistically-significant, dose-dependent increase in relative liver weights noted in females. Histopathological findings occurring with greater frequency and/or severity in α-lipoic acid-treated animals included rarefied periportal hepatocytes often accompanied by cytoplasmic basophilia. The no-observable-adverse-effect level (NOAEL) was considered to be 61.9 mg/kg bw/day. 31.6 and 61.9 mg/kg bw/day No effects on clinical signs, hematology (slight effects in females at 61.9 mg/kg bw/day considered incidental), clinical chemistry, body weight, food intake, reflexes, hearing, dentition status, opthalmological assessments, or urinalysis. No effects on male organ weights (absolute/relative); higher relative kidney weights in females. 121 mg/kg bw/day Slight hypokinesia (45-180 min post-dose) seen in 1 male for 3 days during Wk 4. Incoordination (staggered and stilted gait) noted (within 30-180 min) in several females. Reddish salivation in 1 female and slight clonic convulsions once in another. Lower serum total protein and triglycerides, and slightly higher ALT and GDH. In males, lower serum cholesterol that persisted until study end noted; in females, slightly higher blood urea and cholesterol. Other clinical chemistry findings considered random variations. Higher liver (relative) and kidney weights (absolute/relative) in males; higher absolute kidney weights in females. Higher incidence and slightly greater severity of centrilobular hypertrophy in males. In this group, hepatic microgranulomas (mostly macrophages) tended to be of marginally to slightly greater severity, larger, more frequent, and associated with single-cell necrosis. The mammary gland of females had a marginally higher incidence of diffuse hyperplasia.	GLP-compliant	Cremer *et al.* (2006a)

Table 6.3-1 Summary of toxicological assays of alpha-lipoic acid in rodents (Cont'd)

Endpoint	Test System	Test Material	Dosage or Concentration	Result	OECD/ GLP Compliance	Reference
Chronic oral (diet) study	Rat Sprague-Dawley (Hsd/Win:WU) 40/sex/group[1]	α-lipoic acid (racemic), 99.0 % purity	Diets providing intakes of (mg/kg bw/day) 0* 20 60 180 Administered for 2 years. *Vehicle: 1,2-propylene glycol	NOAEL: 60 mg/kg bw/day No effects on behavior, hearing, hematology, clinical chemistry, urinalysis, relative organ weights, or overall incidence of neoplasms. The following subsets of animals died at or after 15 months due to pneumonia unrelated to treatment: 0 mg/kg bw/day 10 males/9 females 20 mg/kg bw/day 10 males/5 females 60 mg/kg bw/day 3 males/8 females 180 mg/kg bw/day 3 males/7 females Significantly lower food consumption, body weight gains (≥8 wk), and terminal body weights at 180 mg/kg bw/day.	Not specified	Cremer *et al.* (2006b)

[1] The control and high-dose groups each started with 50 rats/sex; 10 rats/sex from each of these groups was sacrificed at 6 months, leaving 40 rats/sex/group to complete the study.

LD_{50}: oral median lethal dose; NOAEL: no-observable-adverse-effect level; ALT: alanine aminotransferase; GDH: glutamate dehydrogenase.

6.4 Reproductive Toxicity

No studies were found in the published scientific literature that specifically evaluated the effects of alpha-lipoic acid on reproduction. However, as a substance produced endogenously by most organisms, and present in the diet at low levels, reproductive toxicity studies of α-lipoic acid would generally be considered a low priority, especially considering that:

(1) well-conducted repeat-dose toxicity tests can in most cases detect substance-related adverse effects on the male and female reproductive tract, and provide an alert for possible effects on fertility (Dent, 2007);

(2) adverse effects of alpha-lipoic acid on the developing offspring might occur only with dosages that far exceed real-life exposures;

However, Hill's intended use is specific to adult dogs that will not be gestating or lactating, hence, although there is no indication of concern with these reproducing animals, it will not be used in reproducing or lactating animals.

6.5 Available Data Inconsistent with the Safety Conclusion

(1) Identify, discuss, and place in context, data and information that are, or may appear to be, inconsistent with your conclusion of GRAS status, regardless of whether those data and information are generally available; or

Published literature that might seem inconsistent with GRAS include published case reports by Loftin and Herold (Loftin and Herold, 2009) of possible a-lipoic acid toxicity in two dogs following accidental consumption of approximately 200 mg/kg bw in a short period of time. The exposure to dl-α-lipoic acid among dogs from its use in canine foods as proposed (150 ppm) is expected to be approximately 2 to 4 mg/kg bw/day, about 50-100 times lower than the levels reported to be toxic.

It has been suggested that cats are more susceptible to a-lipoic acid-related toxicity than humans, dogs, or rats. However, any exposure to a-lipoic acid among cats from collateral consumption of the proposed dog food is expected to be episodic and most likely in the 2 to 3 mg/kg bw/day range. This is 10 to 15 times lower than the 30 mg/kg bw considered the maximum tolerable dose (not lethal) in cats, and 4 to 6 times lower than the 13 mg/kg bw considered to be the no-effect level.

6.6 Safety Conclusion

The maximum tolerated dose of alpha-lipoic acid in dogs was found to be approximately 126 mg/kg body weight (Grunert, RR, 1960) and the proposed LD50 of alpha-lipoic acid in dogs administered orally was reported to be between 400 and 500 mg/kg body weight (Packer L. et al. 1995). In studies published by Hill's on alpha-lipoic acid oral supplementation, dogs were fed

between 0 and 87.7 mg/kg body weight per day (0-4.94 mg/kg body weight per day in Anthony et al, 2021b, 2.5-25 mg/kg body weight/day in Zicker et al, 2010, 0-85 mg/kg body weight per day in Zicker et al, 2002 and 0.31-87.7 mg/kg body weight per day in Pateau-Robinson et al, 2013). The inclusion of alpha-lipoic acid at these concentrations did not pose any health risks to the animals. Evaluations for safety included physical examinations, body weight measurements, measurements of food intake, hematology, serum biochemistry profile. Hill's intends to use a-lipoic acid in dry foods for adult dogs at levels up to 150 ppm (150 mg/kg food or 0.0150%). At these levels, the average lipoic acid exposure in dogs is calculated to be approximately 2.7 mg/kg body weight of the dog/day. This inclusion rate is much lower than the maximum tolerated dose of 126 mg/kg body weight/day and the oral LD50 is 400–500 mg/kg body weight/day. Other published articles on studies of oral alpha-lipoic acid supplementation in canines also did not report adverse events or any health risk to the dogs at the concentrations of inclusion (2 mg/kg body weight/day in Williams DL. 2017, 11 mg/kg body weight/day in Milgram NW et al, 2007). These studies support the use of dl-alpha-lipoic acid as a safe nutritive antioxidant in food for non-gestating, non-lactating adult dogs.

(d) If you view any of the data and information in your notice as exempt from disclosure under the Freedom of Information Act, you must identify the specific data and information; and

Each section designated as [HILL'S CONFIDENTIAL] either in its entirety or in part, of the following sections is Hill's proprietary and confidential information. Hill's requests that this information be exempt from disclosure under the *Freedom of Information Act*.

Section 2.2
Section 2.2.1
Section 2.3
Section 2.4
Section 2.4.1
Table 2.3-1
Table 2.4-1
Table 2.4-2.
Appendix 1
Appendix 2

(e) For non-public, safety-related data and information considered in reaching a conclusion of GRAS status, you must explain how there could be a basis for a conclusion of GRAS status if qualified experts do not have access to such data and information.

Hill's Pet Nutrition is not requesting non-disclosure for any safety information.

§ 570.255 Part 7 of a GRAS notice: List of supporting data and information in your GRAS notice.

(a) In part 7 of your GRAS notice, you must include a list of all of the data and information that you discuss in Part 6 of your GRAS notice to provide a basis for your view that the notified substance is safe under the conditions of its intended use as described in accordance with § 570.250(a)(1).
(b) You must specify which data and information that you list in accordance with paragraph (a) of this section are generally available, and which data and information are not generally available.

Appendices

[HILL'S CONFIDENTIAL]: APPENDIX 1: Specifications for *dl*-alpha-lipoic acid established by Hill's Pet Nutrition

[HILL'S CONFIDENTIAL]: APPENDIX 2: Specifications for *dl*-alpha-lipoic acid established by (b) (4), the intended supplier of material to be used by Hill's

APPENDIX 3: Analytical method developed by Hill's for determination of alpha-lipoic acid in dry pet food

APPENDIX 4: Data validating the analytical method developed by Hill's for determination of alpha-lipoic acid in dry pet food

APPENDIX 5: Results of HPLC analysis of canine foods containing *dl*-alpha-lipoic acid at various levels

APPENDIX 6: Stability data of alpha-lipoic acid in dog foods over 72 weeks.

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Ingredient Specification

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Ingredient Specification

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TECHNICAL DATA SHEET

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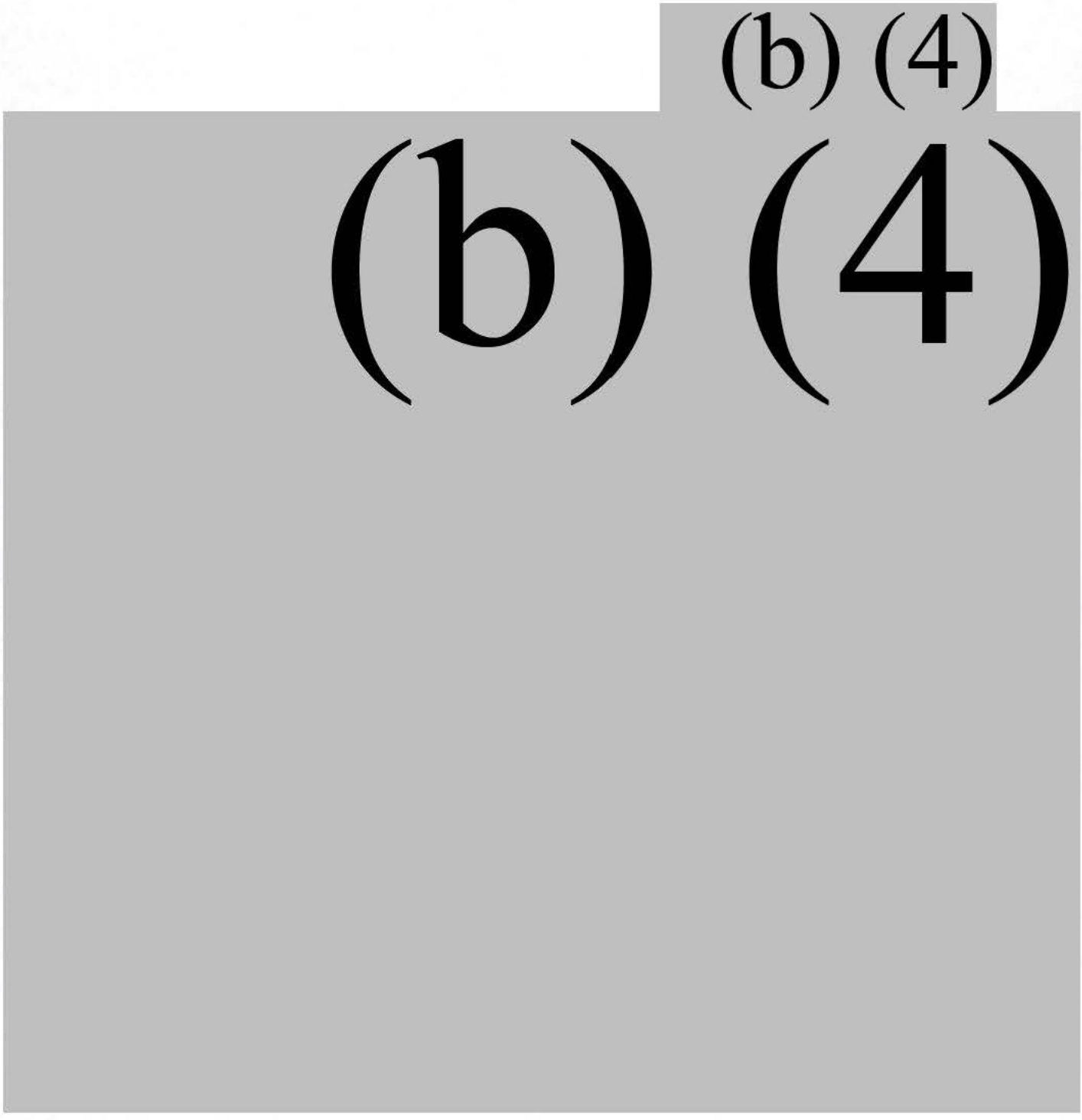
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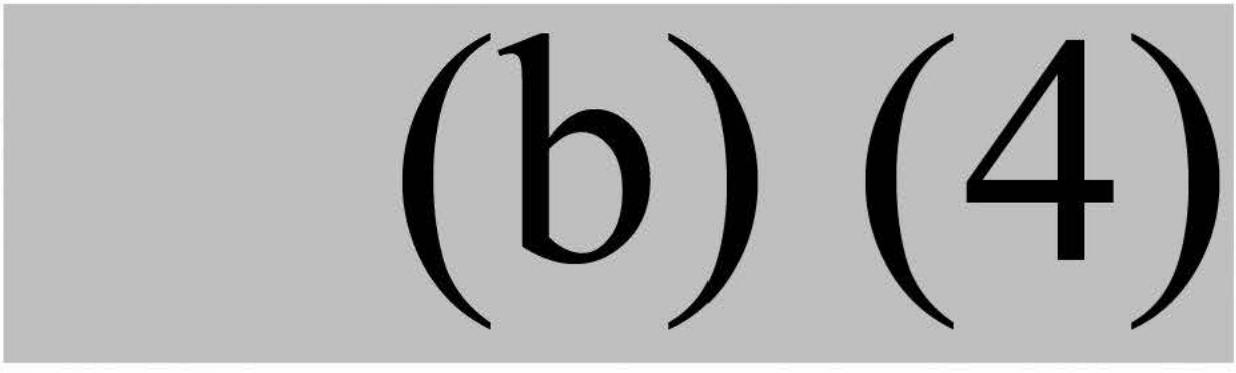
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Preparation of Solutions

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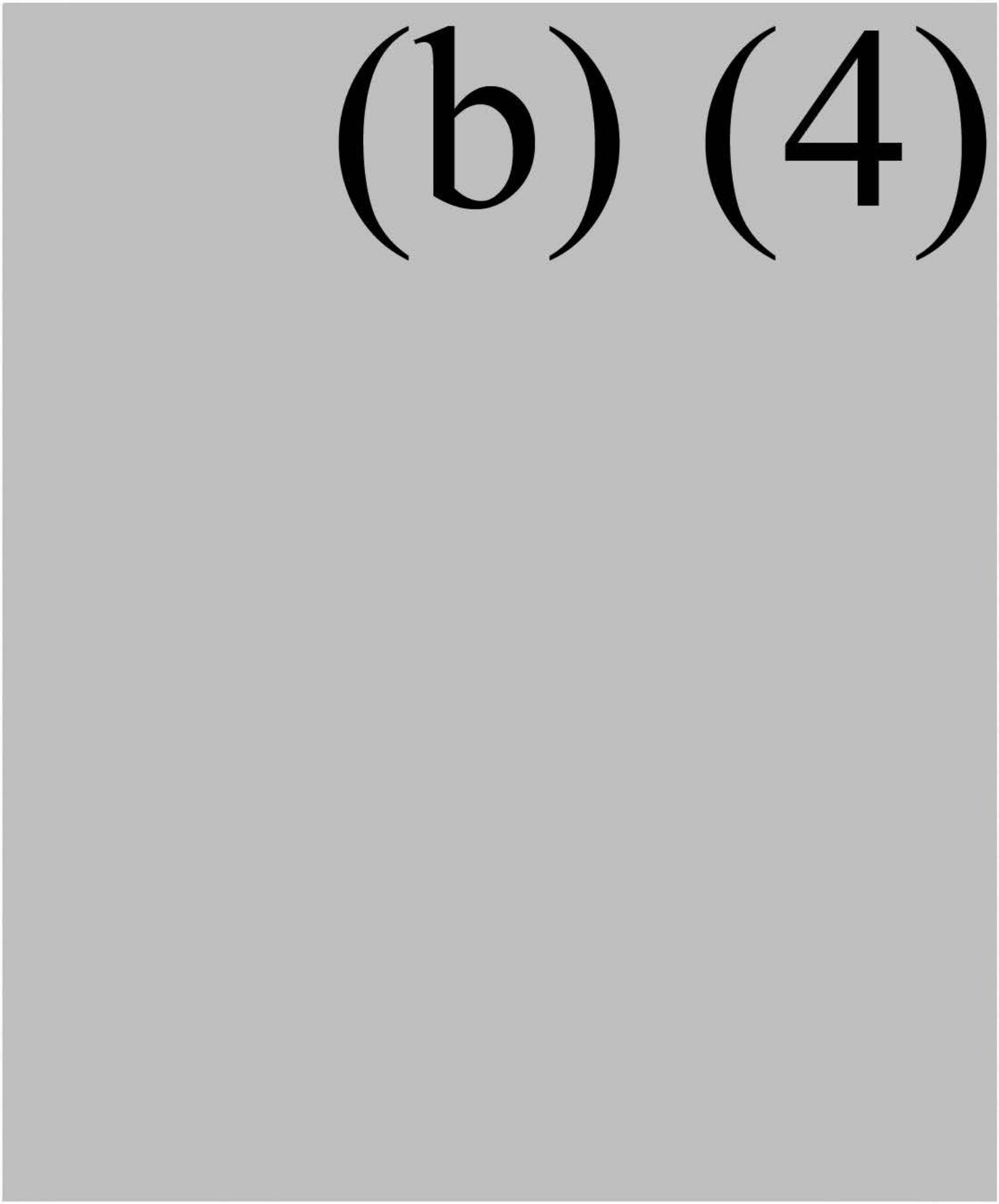

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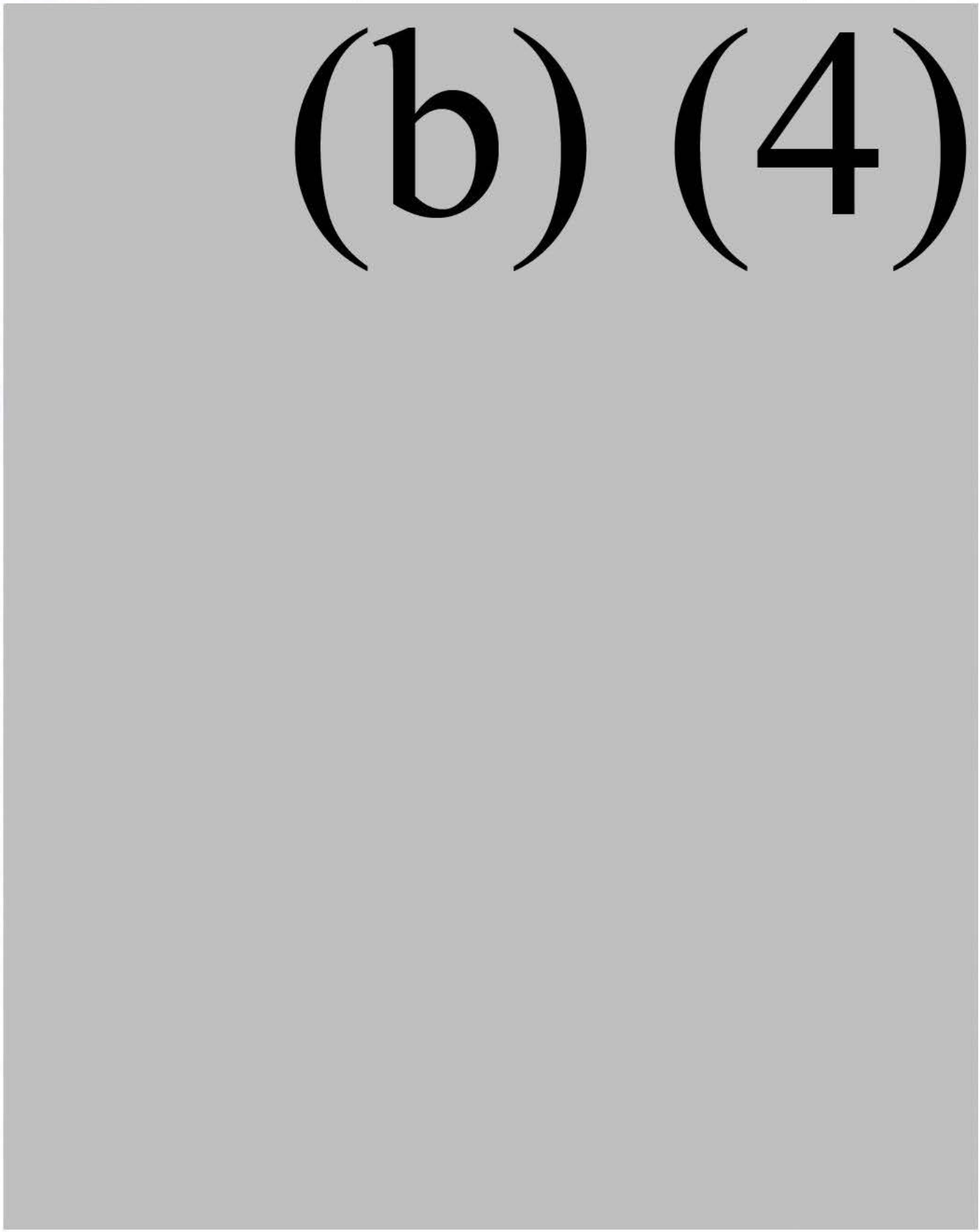

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4. Chromatography

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5. Calculations

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APPENDIX 4: Data validating the analytical method developed by Hill's for determination of alpha-lipoic acid in dry pet food

Summary of parameters evaluated in the validation Hill's analytical method for measuring alpha-lipoic acid in dry canine food

Parameter and Validation Procedure	Outcome
Linearity and Range: Analysis of 7 different calibration standards containing 10-2500 µg/g lipoic acid	Correlation Coefficient ≥ 0.99
Accuracy: Recovery from food samples spiked with lipoic acid (low, medium, high levels) measured	80-120 % Recovery
Repeatability/Reproducibility: Calculate average of the percent coefficients of variance (% CV) Average intraday method precision Analysis in duplicate of 5 extruded pet food samples containing ALA Average interday method precision Analysis of same extruded pet food samples containing ALA, in duplicate on 3 different days	CV < 20 %
Specificity: Compare retention time of derivatized lipoic acid in standards and in samples on each day	**Note:** In the absence of a reference material to use for Quality Control purposes, Hill's uses a food sample that is measured routinely as in house control. Validation of the benchmark for future analysis is based on the average of 12 control samples analyzed over 3 days.

The assay for routine analysis requires: (1) that results expressed in µg/g; (2) use of 7-point calibration daily; (3) a correlation coefficient of 0.98 or better; (4) analysis in duplicate, with a difference of 20% or less between results; (5) analysis of at least 1 spiked sample to demonstrate accuracy, recovery of 80-120 %; (6) analysis of at least 2 in-house control samples with results within 20 % of the value observed during validation.

Formula for calculating spike recovery:

$$\text{Spike Recovery} = \frac{\text{Spike Result}}{\text{(Spike Amount + Sample Result)}} \times 100$$

Results of linear regression analysis of data validating Hill's analytical method for measuring alpha-lipoic acid in dry canine food

Concentration			
µg/g	Day 1	Day2	Day3
0	(b) (4)		
10.00			
50.00			
100.00			
500.00			
1000.00			
2000.00			
2500.00			
Slope			
Intercept			
R^2			

Results of accuracy testing of Hill's analytical method based on recovery of alpha-lipoic acid from spiked samples of dry canine food

	Day 1	Day 2	Day 3
Spike Amount *µg/g*	Average Recovery	Average Recovery	Average Recovery
50.00	92.2%	102.3%	88.5%
100.00	99.5%	102.6%	98.8%
500.00	90.6%	102.5%	98.0%
Overall Average Recovery	**94.1%**	**102.5%**	**95.1%**

Results of inter- and intra-day precision testing of Hill's analytical method for measuring alpha-lipoic acid in dry canine food

Inter-Day Precision (over three days)		
	Concentration *µg/g*	CV
Sample 1	2066.60	1.4%
Sample 2	173.60	7.9%
Sample 3	1214.70	2.1%
Sample 4	3.20	173.2%
Sample 5	1660.10	6.8%

Intra-Day Precision (duplicate analysis per sample)			
Sample Prep Precision			
	Day 1 CV	**Day 2 CV**	**Day 3 CV**
Sample 1	9.22%	6.75%	7.50%
Sample 2	0.88%	4.29%	0.61%
Sample 3	1.62%	9.74%	5.30%
Sample 4	0.00%	2.79%	0.00%
Sample 5	5.62%	6.44%	6.62%
Average CV	**4.34%**	**6.80%**	**5.01%**

Injection Precision			
	CV	**CV**	**CV**
Sample 1	0.01%	0.07%	0.02%
Sample 2	0.03%	0.03%	0.01%
Sample 3	0.00%	0.21%	0.07%
Sample 4	0.00%	0.06%	0.00%
Sample 5	0.09%	0.09%	0.05%
Average CV	**0.04%**	**0.09%**	**0.04%**

APPENDIX 5: Results of HPLC analysis of canine foods containing *dl*-alpha-lipoic acid at various levels

Formula #	Target *dl*-alpha-lipoic acid concentration ppm	Date diet was prepared	HPLC Analysis		
			Days since preparation	Date analyzed	Results µg/g
20409	0	10/09/2000	2	10/11/2000	27.52
			60	12/08/2000	0.00
			72	12/20/2000	10.00
			112	01/29/2001	21.47
		12/21/2000	18	01/08/2001	48.05
			46	02/05/2001	37.78
			92	03/23/2001	37.52
			116	04/16/2001	33.90
			168	06/07/2001	48.11
		05/02/2001	3	05/05/2001	0.00
			42	06/13/2001	10.33
			72	07/13/2001	13.20
			104	08/14/2001	0.00
			188	11/06/2001	0.00
			226	12/14/2001	0.42
20410	150	10/09/2000	2	10/11/2000	144.41
			60	12/08/2000	128.10
			72	12/20/2000	157.79
			112	01/29/2001	149.32
		12/21/2000	18	01/08/2001	147.76
			39	01/29/2001	149.00
			92	03/23/2001	165.09
			116	04/16/2001	145.42
			168	06/07/2001	172.88
		05/02/2001	3	05/05/2001	134.15
			36	06/07/2001	146.38
			72	07/13/2001	145.03
			117	08/27/2001	125.56
			188	11/06/2001	130.27
			226	12/14/2001	135.53

Formula #	Target *dl*-alpha-lipoic acid concentration ppm	Date diet was prepared	HPLC Analysis		
			Days since preparation	Date analyzed	Results µg/g
		10/10/2000	1	10/11/2000	1360.29
			59	12/08/2000	1590.89
			71	12/20/2000	1652.78
			118	02/05/2001	1286.49
		12/21/2000	18	01/08/2001	1375.97
			39	01/29/2001	1414.00
			92	03/23/2001	1482.33
20411	1500		116	04/16/2001	1430.74
			168	06/07/2001	1531.31
		05/02/2001	3	05/05/2001	1335.14
			36	06/07/2001	1307.77
			72	07/13/2001	1332.91
			104	08/14/2001	1474.30
			188	11/06/2001	1403.78
			226	12/14/2001	1339.17

Values expressed as mean values

APPENDIX 6: Stability data of alpha-lipoic acid in dog foods over 72 weeks.

Concentrations of alpha-lipoic acid (ppm) in food were determined periodically over 72 weeks (Week 0, 8, 16, 24, 32, 40, 48, 56, 64 and 72) by high-performance liquid chromatography and were within the range of expected assay sensitivity and production parameters.

Time	75 ppm a-LA food	Avg. of triplicate 75 ppm a-LA food	150 ppm a-LA food	Avg. of triplicate 150 ppm a-LA food	300 ppm a-LA food	Avg. of triplicate 300 ppm a-LA food
Baseline	73.14	73	172.53	172.06	282.52	285.87
Baseline	72.87		171.58		289.21	
Weeks(8)	69.24	70.54	153.72	161.02	302.00	290.74
Weeks(8)	74.24		166.25		278.48	
Weeks(8)	68.14		163.09		291.75	
Weeks(16)	68.92	70.28	167.32	160.57	276.86	288.68
Weeks(16)	73.89		157.13		289.35	
Weeks(16)	68.03		157.25		299.82	
Weeks(24)	67.85	68.45	153.68	155.24	286.37	281.47
Weeks(24)	65.75		157.88		278.15	
Weeks(24)	71.76		154.15		279.88	
Weeks(32)	67.73	68.2	149.09	150.85	278.78	282.21
Weeks(32)	63.98		147.72		284.96	
Weeks(32)	72.89		155.73		282.89	
Weeks(40)	66.83	68.27	156.95	164.58	302.24	294.43
Weeks(40)	69.81		169.14		294.21	
Weeks(40)	68.18		167.66		286.85	
Weeks(48)	62.55	66.03	146.65	147.39	273.57	279.04
Weeks(48)	64.82		150.40		293.00	
Weeks(48)	70.72		145.11		270.55	
Weeks(56)	60.60	63.59	151.37	150.49	278.56	281.61
Weeks(56)	67.58		148.03		291.45	
Weeks(56)	62.58		152.07		274.83	
Weeks(64)	61.95	62.99	150.33	148.93	288.27	280.7
Weeks(64)	64.22		148.11		291.01	
Weeks(64)	62.80		148.34		262.81	
Weeks(72)	63.11	62.56	133.29	139.85	253.92	263.65
Weeks(72)	61.17		146.40		266.67	
Weeks(72)	63.41		N/A		270.37	

Figure below shows mean alpha-lipoic acid concentration in ppm in the different Foods analyzed by High-Pressure Liquid Chromatography-Control, Food 1, Food 2 and Food 3 have 0 ppm, 75 ppm, 150 ppm and 300 ppm of alpha-lipoic acid respectively