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Jeannie Perron, JD, DVM

Covington & Burling LLP
One CityCenter
850 Tenth Street, NW
Washington, DC 20001-4956
T +1 202 662 5687
jperron@cov.com

June 30, 2016

VIA FEDERAL EXPRESS

Dr. David Edwards
Food and Drug Administration
Division of Animal Feeds (HFV-220)
Office of Surveillance and Compliance
Center for Veterinary Medicine
7519 Standish Place
Rockville, Maryland 20855

Re: GRAS Notice for marigold extract (*Tagetes erecta*) as a lutein source in dog and cat diets

Dear Dave:

On behalf of Royal Canin U.S., Inc., I am hereby submitting the following materials:

1. One hard copy of a June 23, 2016 letter from Royal Canin USA Inc. (Royal Canin) to Dr. Louis Carlacci explaining in response to FDA's Memorandum of the February 1, 2016 Teleconference the changes made to the GRAS notification entitled "GRAS Exemption Claim for Marigold Extract Derived from *Tagetes erecta* for Use in Diets and Treats for Dogs and Cats". A copy of the Memorandum is attached to the letter;

2. A copy of the revised notification itself and all references;

3. A set of redacted copies of the notification and those references that contained confidential/trade secret information that is protected from disclosure pursuant to 5 U.S.C. § 552(b)(4), 18 U.S.C. § 1905 and 21 C.F.R. § 20.61. These documents have been redacted to mask the confidential information; and

4. A disk that contains electronic copies of all of the documents I am submitting in hard copy.

Please file-stamp the supplementary copy of the June 23, 2016 letter from Royal Canin and GRAS Notice and return the file-stamped copy to me in the enclosed, self-addressed, pre-paid Federal Express envelope.

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If you need any additional information, please do not hesitate to contact me. Your assistance is sincerely appreciated.

Respectfully submitted,

Jeannie Perron, JD, DVM

Attachments

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ROYAL CANIN

June 23, 2016

Center for Veterinary Medicine
Food and Drug Administration
7519 Standish Place, HFV-1
Rockville, MD 20855
240-402-7002

Dear Dr. Louis Carlacci,

By way of this correspondence, we wish to address the changes/corrections requested to the GRAS notification entitled 'GRAS Exemption Claim for Marigold Extract Derived from *Tagetes erecta* for Use in Diets and Treats for Dogs and Cats' included in the Memorandum of February 1, 2016 Teleconference (attached).

In the Chemistry, Manufacturing and Controls section the following changes have been made (Bold text are FDA comments from February 1, 2016 Teleconference (see attached)):

1) **For the recommended use level of marigold extract oleoresin (the subject of the GRAS notice).** Information concerning the recommended use level of marigold extract (also termed marigold oleoresin) has been added to Section I.B.2, Section IV.A, and Table 11. For instance, the following paragraph has been added to Section I.B.2, and similar language and information has been included in Section IV.A and Table 11.

(b) (4)

2) **For the composition of the oleoresin, which includes among other things the levels of total carotenoids and lutein. Published studies supporting the oleoresin composition.** We have performed an extensive literature search to identify published studies that detail the composition of marigold oleoresin. Very little

Royal Canin USA
500 Fountain Lakes Blvd
Suite 100
St. Charles, MO 63301
www.royalcanin.us

T 636.926.0003
CS 800.592.6687
Efax 636.926.3859

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information exists in the published literature, however citations have been included that support the composition of free fatty acids, gums and resins in marigold extract as reported in Table 2 (See Section II.C).

3) **Specifications for the marigold extract oleoresin provided by suppliers. (Specifications consist of tests, acceptance criteria and validated analytical methods.)** Specification information including the tests performed, acceptance criteria, and methodology used have been included in the Section II.B. Furthermore, a description of the supplier quality plan for the development of marigold oleoresin (extract) has been included. All quality plans, specifications, and methods described in this GRAS notification are company confidential.

4) **Validated methods for the determination of lutein in the marigold extract oleoresin, the pet food ingredient containing the oleoresin, and pet food.** Section II.E was added which details the validated methodology used to measure xanthophyll and lutein concentration in marigold extract/oleoresin (the pet food ingredient) and to measure lutein concentration in the pet food.

5) **Information on the stability of the marigold extract oleoresin, the pet food ingredient containing the oleoresin, and pet food containing the oleoresin. For, example, stability information for the pet food ingredient should support the 12 month shelf life.** Section II.D was added which demonstrates the stability of lutein in marigold extract (the pet food ingredient) and in pet food for the 12 month (dry) and the 24 month (wet) shelf life. This section details stability studies that were performed on marigold extract oleoresin and on the lutein from marigold extract in the finished product (dog and cat food). Together, these data show both, that xanthophyll content (including lutein) in marigold extract (Cuatroxan) is stable for at least 6 months from the day of production, and that lutein is stable for the entire shelf life (and even well past the intended shelf life) of wet and dry cat and dog foods.

6) **Food grade specifications for each component of the pet food ingredient.** A statement has been added in section II.B.2 stating 'The ingredients, including marigold extract, used during manufacturing are all feed grade not food grade as per GMP requirements'.

7) **An indication that the use of rosemary extract in the pet food ingredient is consistent with that of a flavor.** Details were added to Sections II.B.1 and II.C.9 which illustrate that the inclusion amount of rosemary extract in the dog and cat food is consistent with its approved use as a flavorant. As stated in Section II.B.1, at inclusion rates of 1300 ppm (Cuatroxan) and 2300 ppm (Nutri-Gold Yellow) in pet food (see Section IV), the amount of rosemary extract in the pet food would be no

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more than [redacted] (b) (4) respectively, which is within the acceptable level to impart a flavor.

8) **CVM asked the firm to provide information on pesticide residue levels in the oleoresin. The information submitted in the notice contains limited information on the type and concentration of commonly used pesticides.** Information concerning the pesticides residue levels in the oleoresin has been added in Section II.B). Please see the tables in this section.

In the Utility section the following changes have been made:

9) **In the GRAS exemption claim, stating only that marigold extract is a source of lutein is not acceptable. This is only sufficient for essential nutrients. The exemption claim should include a demonstrated food use.** The GRAS exemption claim (see Section I) was modified to include information on the demonstrated pet food use of marigold extract as a source of antioxidant activity.

10) **Stating that marigold extract replaces lutein from other ingredients no longer used in pet food does not support utility.** It was not the intent to imply that the presence of lutein from other ingredients in pet food supported utility. Rather, that lutein has been a common component of foods for dogs and cats for many years without observed ill-effect. We believe that this speaks to the safety of lutein for dogs and cats, and that the recommended use of lutein from marigold extract described in this GRAS notification does not exceed the levels previously provided by once-common ingredients such as corn gluten meal. The utility section (See Section V) has been re-written to provide focus on the antioxidant properties that marigold extract and lutein derived from marigold extract possess. In addition, the nutritive effect of these antioxidant properties for dogs and cats is detailed.

11) **The antioxidant properties need to be demonstrated to support a nutritive effect. The cited immune challenge studies do not appear to demonstrate a nutritive effect. Reviewing the canthaxanthin GRAS notice may be helpful in determining how to demonstrate a nutritive effect; the intended use in both notices is similar.** After reviewing the canthaxanthin GRAS, the utility section (See Section V) has been re-written to provide focus on the antioxidant properties that marigold extract and lutein derived from marigold extract possess including supporting cellular health. We outline studies that demonstrate that lutein derived from marigold extract supports immune system health. The nutritive effect of these antioxidant properties for dogs and cats is highlighted in Section V. In addition to the utility section, a number of sections and formatting was updated to be consistent with the canthaxanthin GRAS notice.

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12) **Studies using a mixed test article may not be helpful in demonstrating intended use; in that case it is difficult to attribute the effect to a single substance.** Section V.D.5 provides the rational for use of studies that contain mixed test articles in demonstrating the intended use. As described in this section, the studies that demonstrate utility of lutein in dogs and cats use a mixed test article that contains lutein and zeaxanthin, purified from marigold petals. This is comparable to marigold extract, the subject of this GRAS Notification, which is also composed of both lutein and zeaxanthin.

Best Regards,

Patricia G. Keller, Ph.D.

Patricia G. Keller, M.A., Ph.D.

Director of Regulatory Affairs, U.S.A.
Royal Canin USA, Inc.

Royal Canin USA
500 Fountain Lakes Blvd
Suite 100
St. Charles, MO 63301
www.royalcanin.us

T 636.926.0003
CS 800.592.6687
Fax 636.926.3859

BEST COPY AVAILABLE

M000043Z0001
Marigold extract oleoresin
Royal Canin U.S., Inc.

Memorandum of February 1, 2016 Teleconference

CVM Participants	Firm Participants
Mr. Geoffrey Wong, HFV-224	**Royal Canin USA, Inc.**
Dr. Louis Carlacci, HFV-224	Dr. Patricia Keller (via phone) [1]
Ms. Charlotte Conway, HFV-228	
Dr. Angela Betancourt, HFV-224	**Kemin** [2]
Dr. David Edwards, HFV-220	Dr. Andrew Yersin (via phone)
	Regulatory Discretion, Inc. [3]
	Dr. David A. Dzanis (via phone)
	Covington & Burling [4]
	Dr. Jeanne Perron (via phone)

Purpose: CVM is providing the notifier with information on the results of CVM's cursory review of the notice prior to filing the notice.

Chemistry, Manufacturing and Controls (CMC)
CVM stated that the following information is missing from the notice.

1) Recommended use level of marigold extract oleoresin (the subject of the GRAS notice).
2) Composition of the oleoresin, which includes among other things the levels of total carotenoids and lutein. Published studies supporting the oleoresin composition.
3) Specifications for the marigold extract oleoresin provided by suppliers. (Specifications consist of tests, acceptance criteria and validated analytical methods.)
4) Validated methods for the determination of lutein in the marigold extract oleoresin, the pet food ingredient containing the oleoresin, and pet food.
5) Information on the stability of the marigold extract oleoresin, the pet food ingredient containing the oleoresin, and pet food containing the oleoresin. For, example, stability information for the pet food ingredient should support the 12 month shelf life.
6) Food grade specifications for each component of the pet food ingredient.
7) An indication that the use of rosemary extract in the pet food ingredient is consistent with that of a flavor.

Dr. Andrew Yersin asked for clarification and / or confirmation from CVM on what was being asked. CVM responded. The firm was asked to provide published information on the identity of marigold extract, including composition and stability. This information is useful to establish a

[1] 500 Fountain Lakes Boulevard, Suite 100, St. Charles, Missouri, 63301; T: 636-926-1068.
[2] 2100 Maury Street, Des Moines, Iowa 50317; T: 515-559-5505; F: 515-559-5232.
[3] 16256 Ravenglen Road, Santa Clarita, California 91387; T: 661-645-4959; F: 661-251-3203.
[4] One City Center, 850 Tenth Street Northwest, Washington, D.C. 20001; T: 202-662-5687.

Royal Canin USA
500 Fountain Lakes Blvd
Suite 100
St. Charles, MO 63301
www.royalcanin.us

T . 636.926.0003
CS . 800.592.6687
Efax . 636.926.3859

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qualitative and quantitative relationship between the marketed products and the substance of the GRAS notice.

Target animal safety:
CVM asked the firm to provide information on pesticide residue levels in the oleoresin. The information submitted in the notice contains limited information on the type and concentration of commonly used pesticides.

Dr. Andrew Yersin asked for clarification from CVM on what was being asked. CVM responded.

Utility:
CVM stated that the following are missing from the notice:

1) In the GRAS exemption claim, stating only that marigold extract is a source of lutein is not acceptable. This is only sufficient for essential nutrients. The exemption claim should include a demonstrated food use.
2) Stating that marigold extract replaces lutein from other ingredients no longer used in pet food does not support utility.
3) The antioxidant properties need to be demonstrated to support a nutritive effect. The cited immune challenge studies do not appear to demonstrate a nutritive effect. Reviewing the canthaxanthin GRAS notice may be helpful in determining how to demonstrate a nutritive effect; the intended use in both notices is similar.
4) Studies using a mixed test article may not be helpful in demonstrating intended use; in that case it is difficult to attribute the effect to a single substance.

CVM provided the GRAS number for canthaxanthin as AGRN 17.

Based on the information on the results of CVM's cursory review, CVM stated that the notice is not acceptable for filing. Therefore, CVM recommended that the additional needed information be included in the GRAS notice.

Meeting concluded with Dr. Perron indicating that they will discuss amongst themselves on how to proceed.

Louis Carlacci, Ph.D.
Ingredient Safety Team, HFV-224

Royal Canin USA
500 Fountain Lakes Blvd.
Suite 100
St. Charles, MO 63301
www.royalcanin.us

T 636.926.0003
CS 800.592.6687
Efax 636.926.3859

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GRAS Exemption Claim for Marigold Extract Derived from *Tagetes erecta* for Use in Diets and Treats for Dogs and Cats

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Table of Contents

BEST COPY AVAILABLE

BEST COPY AVAILABLE

BEST COPY AVAILABLE

List of Tables

List of Appendices

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GRAS Exemption Claim for Marigold Extract Derived from *Tagetes erecta* for Use in Diets and Treats for Dogs and Cats

I. GRAS EXEMPTION CLAIM

Marigold extract, which contains a significant amount of the yellow pigment lutein, has been approved for and commonly used as a colorant or flavorant in pet food. In addition to its pigmentation qualities, lutein is a potent antioxidant. Studies have shown that dietary lutein has a powerful nutritional benefit to dogs and cats, including promoting healthy immune response and protecting cells from oxidative damage. For this reason at the request of Royal Canin U.S., Inc. (Royal Canin), a panel of independent scientists, qualified by their experience and scientific training to evaluate the safety of ingredients used in animal feed, including pet food (Expert Panel), was convened to evaluate the pertinent data and information and to determine whether marigold extract (*Tagetes erecta*) would be Generally Recognized as Safe (GRAS) for use in dog and cat food. Under the conditions of intended use marigold extract would be added to levels of approximately 2300 ppm or enough marigold extract to ensure lutein is present in diets and treats for dogs and cats at levels of up to 5 mg per 1000 kcal metabolizable energy.

The Expert Panel consisted of the below-signed qualified scientific experts: David A. Dzanis, DVM, PhD, DACVN, CEO of Regulatory Discretion, Inc.; David J. Maggs, BVSc, DACVO, Professor of Comparative Ophthalmology at the University of California-Davis; and Helen Clegg, PhD, Safety Scientist Toxicologist, Mars Petcare, Inc. *Curricula vitae* reflecting the Panel members' qualifications for evaluating the safety of food ingredients are provided in Appendix A. Following independent and collective critical evaluation of the data and information, the Expert Panel concluded that under the conditions of intended use as a nutritional source of the antioxidant lutein in diets and treats for dogs and cats at levels sufficient to supply no more than 5 mg lutein per 1000 kcal metabolizable energy (1300 ppm for Cuatroxan and 2300 ppm for Nutri-Gold Yellow), marigold extract (*Tagetes erecta*) is GRAS, based on scientific procedures. A summary of the basis for the Expert Panel's conclusion is provided below.

I.A Common Name of the Substance

The common name of the notified substance is marigold extract.

I.B Conditions of Intended Use in Pet Food

I.B.1 Intended Use of Marigold Extract in Pet Food and Level of Use

Marigold, like many plants, contains high levels of the carotenoids, particularly the yellow pigment lutein (Section II.C). For this reason marigold extract has historically been used as a color additive in chicken feed and a natural flavor for human food and animal feed. In the United States, preparation of marigold as an oil from *T. patula L.*, *T. erecta L.*, or *T. minuta L.*

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(*T. glandulifera Schrank*) is approved for safe use in human food either as a natural flavoring substance or as a substance used in conjunction with flavoring substances under 21 C.F.R. § 172.510. These substances are also permitted for use in animal feed, including dog and cat food, as a "natural flavor" under 21 C.F.R. § 501.22(a)(3).

Royal Canin intends to use marigold extract as a nutritional source of the antioxidant lutein in diets and treats for dogs and cats at a level sufficient to provide no more than 5 mg lutein per 1000 kcal metabolizable energy (ME). The marigold extracts of primary commercial interest to Royal Canin are produced by Kemin Industries, Inc. under the trade name Cuatroxan®, and Nutri-AD International NV, under the trade name Nutri-Gold Yellow, although Royal Canin may also use marigold extract manufactured by other suppliers in its products (See Appendix B). The recommended use of marigold extract in pet food is dependent on the concentration of lutein in the marigold extract, the type of diet (i.e. maintenance or high energy), and the amount of (b) (4) Using the average amount of lutein in Cuatroxan and Nutri-Gold Yellow and (b) (4), the recommended use of marigold extract is 1300 ppm for a high energy diet and 2300 ppm for a maintenance diet (See Section IV). Regardless of the commercial source of the marigold extract, the extract will be derived from *T. erecta* and the use level will be determined in order to ensure that the amount of lutein in the pet food does not exceed 5 mg per 1000 kcal ME.

Marigold meal and extract from *T. erecta* (also known as Aztec Marigold) is approved as a color additive for use in chicken feed under 21 C.F.R. § 73.295 ["*Tagetes* (Aztec marigold) meal and extract"]. The color additive regulation is not applicable to Royal Canin's intended use both because the regulation is only applicable to chicken feed and because Royal Canin is using marigold extract for nutritive purposes, rather than as a color additive. Royal Canin submitted its intention to use marigold extract as a nutritional source of lutein in dog and cat food to the US Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition's (CFSAN) Office of Food Additive Safety, Division of Petition Review to ask whether Royal Canin would need to seek a color additive approval to cover that use. CFSAN confirmed that Royal Canin did not need to obtain a color additive approval to use marigold extract as a nutritional source of lutein in pet foods. (See Appendix C)

Preparation of marigold as an oil from *T. patula L.*, *T. erecta L.*, or *T. minuta L.* (*T. glandulifera Schrank*) is also approved for safe use in human food either as a natural flavoring substance or as a substance used in conjunction with flavoring substances under 21 C.F.R. § 172.510. The substances listed in this part, including marigold oil as described above, are also permitted for use in animal feed, including dog and cat food, as a "natural flavor" under 21 C.F.R. § 501.22(a)(3).

Moreover, marigold extract has been the subject of several GRAS Notifications for use in human food and CFSAN has had no questions with respect to any of these notifications. See, *e.g.*, the June 14, 2004 CFSAN letter regarding the Kemin Foods, L.C. GRAS Notice 140 for a lutein preparation (crystalline lutein) from *Tagetes erecta* marigold flowers at levels varying from 0.3 to 3.0 mg per reference amount customarily consumed (RACC), depending on the particular food. The FDA had no questions regarding GRN 110 by Cognis Corporation for a marigold extract use level to provide lutein esters up to 6.0 mg per serving, depending on the type of food. GRAS Notice 291, filed on behalf of Industrial Organica S.A. de C.V., notified CFSAN that the company had determined marigold-derived crystalline lutein to be GRAS at levels from 0.3 to 3.0 mg/RACC, depending on the type of food. OmniActive Health Technologies Ltd. filed

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GRAS Notice 385 for a lutein preparation from *Tagetes erecta* marigold flowers, also at levels from 0.3 to 3.0 mg/RACC, depending on the type of food. For infant formula, Abbott Nutrition filed GRAS Notification 390 for marigold-derived suspended lutein at 210 micrograms lutein per liter of formula. Finally, Industrial Organica S.A. de CV notified FDA of its determinations that marigold-derived lutein, lutein diacetate, and lutein esters are GRAS at levels of 0.3 to 3.0 mg/RACC (*See* GRAS Notifications 432, 542 and 543). These are discussed in greater detail later in this document, however in all cases, FDA had no questions with respect to these GRAS determinations.

Royal Canin's proposed use of marigold extract is as a nutritional source of lutein. Many pet food ingredients contain inherent levels of lutein. Egg yolks contain up to 0.3 mg lutein per yolk. Vegetables, including spinach, kale, broccoli, Brussel sprouts, cabbage and green beans can provide lutein. However, the most common source of lutein in pet foods is from corn gluten meal.

Marigold extract also contains a significant amount of lutein and because of the lutein derived-yellow pigmentation it has been commonly used a food and feed colorant. In addition to lutein's pigmentation qualities, researchers have begun to focus on its antioxidant properties. Lutein is a natural, fat soluble antioxidant and has been identified as a potent scavenger of free radicals (Sindhu et al., 2010). Several studies have recently shown that dietary lutein has nutritive benefits in cats and dogs, namely promoting healthy immune response and protecting health cells from oxidative damage. It is for this reason that the subject of this GRAS notification is for use of marigold extract as a nutritional source of the antioxidant lutein in diets and treats for dogs and cats.

I.B.2 Level of Dietary Exposure Resulting from Royal Canin's Intended Use

The safety of marigold extract was evaluated by calculating the maximum dietary exposure to marigold extract that will result from Royal Canin's use on a milligram per kilogram body weight per day (mg/kg.bw/day) basis.

(b) (4)

The presumed safe intake of lutein as determined by the National Research Council of the National Academies (NRC) is 1.8 mg/kg.bw/day for dogs and 7.2 mg/kg.bw/day for cats. At the levels of inclusion in these above examples, dogs at maintenance eating the lower energy food in the example above will consume between 0.22 and 0.60 mg lutein/kg.bw/day, whereas adult dogs with greater energy needs (e.g., working dogs) eating the higher energy food above will consume between 0.47 and 1.27 mg lutein/kg.bw/day.

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Puppies will consume more lutein per kilogram body weight than adult dogs. At the maximum rate of inclusion of marigold extract (5 mg lutein/1000kcal ME), a 17 kg large-breed puppy will consume 0.57 mg lutein/kg.bw/day, while a 1.5 kg small-breed puppy will consume 1.26 mg lutein/kg.bw/day. Similarly, kittens will consume more lutein per kilogram body weight than adult cats. At the same concentration of marigold extract in a cat food, a 0.8 kg kitten will consume 1.09 mg lutein/kg.bw/day.

In all cases the estimated intake of lutein from Royal Canin's proposed use of marigold extract is well below the presumed safe intake level determined by NRC.

I.C Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the FDA upon request. Should the FDA have any questions or additional information requests regarding this notification, Royal Canin will supply these data and information.

II. DETAILED INFORMATION ABOUT THE SOURCE AND IDENTITY OF THE SUBSTANCE: Marigold Extract

II.A Source and Identity

Marigold extract is derived from the flowers of *Tagetes erecta*. Although not commonly consumed in North American, Marigold petals have a long history of safe use in human diets. In 1917 "the Herbalist" indicated that Marigold petals were a source of vitamin C and phosphorus (Smith, 1973). Because of their color and nutritive benefits, marigold petals have been used in a variety of culinary applications including in salads and with eggs (Smith, 1973). The petals are often substituted in place of saffron and tarragon in casseroles, vegetable dishes, and in venison and seafood recipes (Smith, 1973). In South America, marigold petals are made into a paste and are a main component of a potato dish called ocopa. Additionally because of their nutritional value, the petals have been routinely fed to farm animals, such as pigs and chickens. Marigold petals from which marigold extract is derived have a long history of safe use in both human and animal diets.

Marigold extract is a mixture of various compounds; thus, the safety of each substance must be considered. Analysis of Cuatroxan marigold extract indicates it consists of carotenoids (of which 70% are lutein); C_{21}-C_{31} alkanes; free fatty acids; and gums and resinous matter (Table 2). There is no reason to believe that marigold extract from another supplier would vary significantly from this composition. However, two factors are noted, that could have an impact on the composition of the extract: (1) the method of extraction, *e.g.*, the time and temperature and the specific solvent or any enzymes used;[1] and (2) the plant's stage of development, as well as the climate

[1] The extract approved under 21 C.F.R. § 73.295 is simply described as the hexane extract of the flower petals. The natural flavoring substance approved under 21 CFR § 172.510 for use in human food is defined far more broadly. The method of production can affect the composition

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and season (Héthély et al., 1986; Bosma et al., 2002). These factors could have an impact on the composition of the extract, but based on our research, we are confident that we can consider the Cuatroxan profile to be representative of the marigold extract produced by other suppliers.

II.B Method of Manufacturing

II.B.1. Marigold Oleoresin (Extract)

The manufacturing process for marigold oleoresin (also called marigold extract), is exemplified by the flow process of Kemin (Table 1). The process is not materially different for other sources of marigold extract. Briefly, hybridized marigold flowers from various geographical locations are sourced and utilized in the process. The extract is produced by hexane extraction of the dried marigold leaves and the resulting material is termed "oleoresin", which is a concentrate of carotenoids, namely, xanthophylls including lutein. The typical fat content of the oleoresin is approximately 98%. (b) (4)

of the extract. For example, in distillation (versus extraction), we would expect to have a higher ratio of volatile to non-volatile substances than the ratio in a hexane extract.

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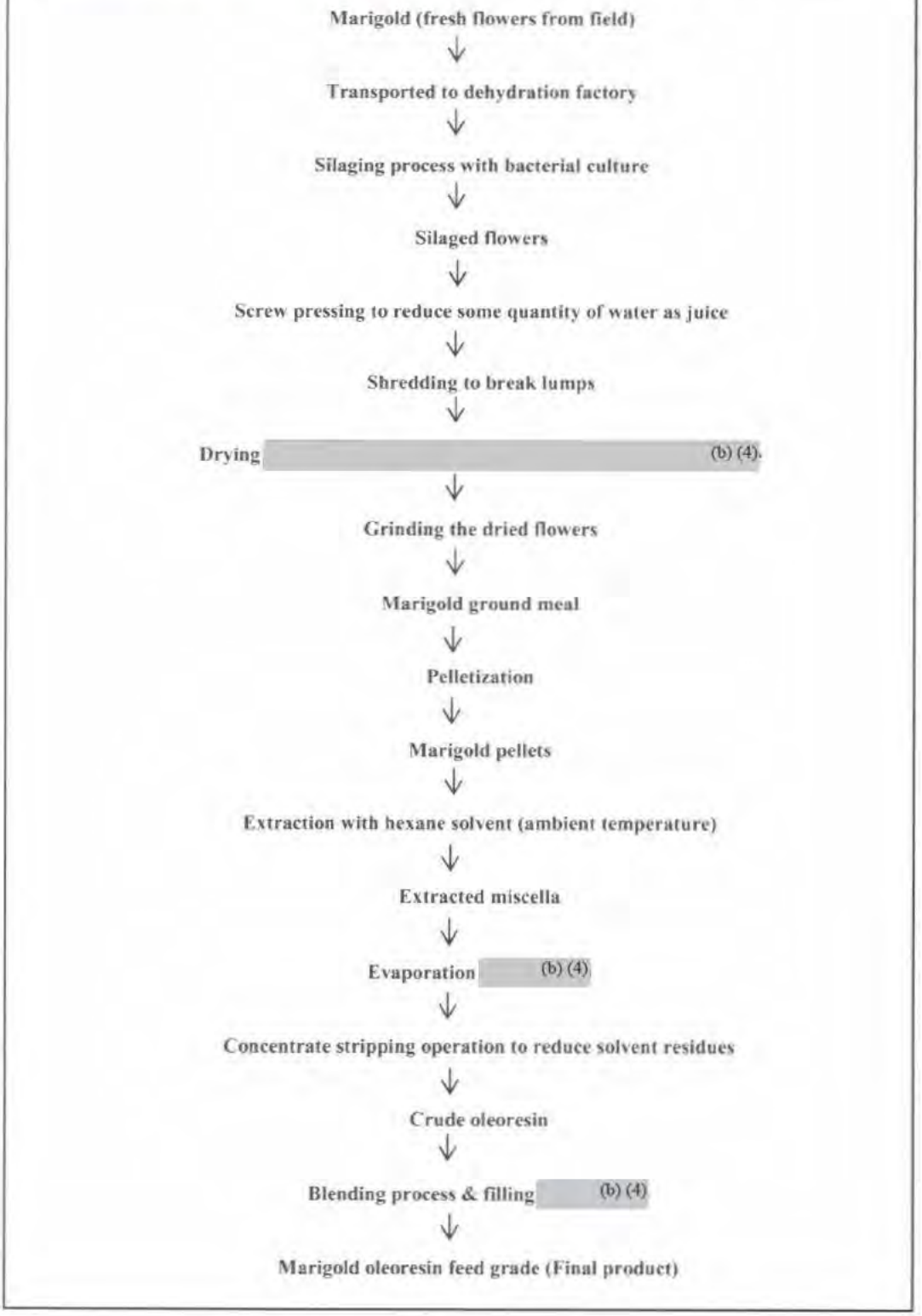
Table 1. Flow Process for Marigold Oleoresin – Feed Grade



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Prior to its use in the manufacturing process, it is critically important to confirm the marigold oleoresin supplier specification and certificate of analysis. An example of supplier documentation (confidential), including the specifications used to establish acceptable levels of lutein, ethoxyquin, BHA/BHT, and dioxins and PCBs is shown below.

G. PRODUCT CHARACTERISTICS

(b) (4)

In addition to supplier testing, Kemin has also established a quality testing plan to ensure the quality of Cuatroxan. The quality plan is confidential, however it may include (among other things) testing of numerous heavy metals, pesticides, dioxin, impurities, and bacteria (see below).

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Hazard	Method	Alert Level
Heavy Metals	AOAC or equivalent	30 ppm Arsenic 10 ppm Lead 0.2 ppm Mercury 10 ppm Cadmium
Pesticides	QuEChERS	No known regulatory limits
Dioxin	EPA 1613	200 ppt
Impurities	USP <467> Residual Solvents	290 ppm Hexane
Bacteria	TPC	2x10^6 CFU/g Salmonella neg/25g

(b) (4)

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II.B.2. Method of Addition to the Pet Food Diet

(b) (4)

II.C Composition of Marigold Extract

Compositional analysis of Cuatroxan by the supplier identified the constituents as per Table 2. The supplier also confirmed that the typical lutein content was approximately 70% of the total carotenoids in the Cuatroxan formula.

This GRAS specifically addresses marigold extract however the additional ingredients that are added during the manufacturing process as carriers, processing aids or stabilizers are also considered (Table 3). The extraction procedure for marigold remains similar and therefore the relative composition of the chemicals within the marigold extract compared to the lutein fraction are not expected to differ significantly.

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Table 2: Composition of Marigold Oleoresin (Extract)

(b) (4)

Table 3: Composition of CUATROXAN® brand Dry

(b) (4)

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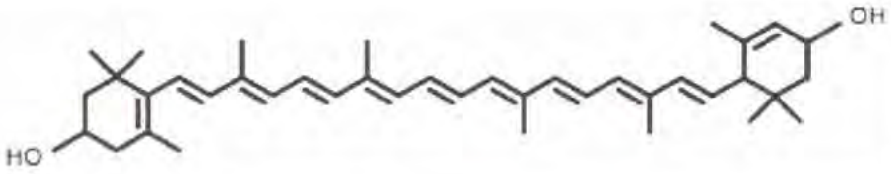
II.C.1 Lutein

Lutein is a xanthophyll and one of 600 known naturally-occurring carotenoids. Lutein is synthesized by plants, microalgae and some photosynthetic microorganisms. It occurs naturally with high levels in green leafy plants such as kale, dandelion leaves, nasturtium (watercress), turnip greens, spinach, Swiss chard, peas, lettuce, and many others, including the marigold flower, *Tagetes erecta* (full discussion in section VI.A.1). Lutein is present in plants as fatty-acid esters, with one or two fatty acids bound to the two hydroxyl-groups. Lutein is an oxycarotenoid that contains 2 cyclic end groups (one β- and one ε-ionone ring) and the skeletal C_{40} isoprenoid structure, which is common to all carotenoids. The principal natural stereoisomer of lutein is (3*R*, 3'*R*, 6'*R*) - β, ε-carotene-3,3'-diol. Lutein, which is lipophilic, is insoluble in water but soluble in fats, and has a melting point of 190 °C. Detailed descriptions of lutein are provided in Table 4.

Table 4. Lutein Description

Chemical Names:	**4-[18-(4-Hydroxy-2,6,6-trimethyl-1-cyclohexenyl)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-3,5,5-trimethyl-cyclohex-2-en-1-ol; Xanthophyll; Bo-Xan; β,ε-carotene-3,3'-diol; Vegetable lutein; Vegetable luteol; all-*trans*-(+)-xanthophyll; all-*trans*-lutein; Luteine**
Chemical Formula:	**$C_{40}H_{56}O_2$**
Molecular Weight:	**568.88**
CAS No.:	**127-40-2**
Chemical Structure:	

As a food additive, lutein has the identifier E number E161b (INS number 161b) and is extracted from marigold petals (*Tagetes erecta*). It is approved for use in the EU, Australia, and New Zealand (See Australia New Zealand Food Standards Code, 2015).

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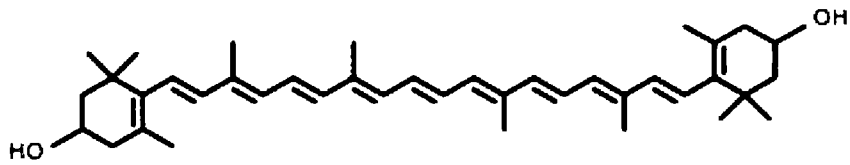
II.C.2 Other Carotenoids (i.e., Zeaxanthin)

Lutein and zeaxanthin have identical chemical formulas and are isomers. The only difference between them is the location of the double bond in one of the end rings. This difference gives lutein three chiral centers whereas zeaxanthin has two. Other than lutein, zeaxanthin is the only other carotenoid present in a notable concentration in marigold extract. Other carotenoids, including cis- and trans- lycopene, Alpha, beta cryptoxanthin, alpha carotene and cis- and trans-B-Carotene, were not detected by HPLC analysis of marigold oleoresin. Detailed descriptions of zeaxanthin are provided below in Table 5.

Table 5. Zeaxanthin Description

Chemical Names	4-[18-(4-hydroxy-2,6,6-trimethyl-1-cyclohexenyl)-3,7,12,16-tetramethyl-octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]-3,5,5-trimethyl-cyclohex-3-en-1-ol; β,β -carotene-3,3'-diol; zeaxanthin
Chemical Formula	$C_{40}H_{56}O_2$
Molecular Weight	568.88
CAS No.	144-68-3
Chemical Structure	

II.C.3 C_{21}-C_{31} Alkanes

Chemical analysis of the marigold extract identified a number of long chain alkanes that are typically associated with plant waxes produced by all terrestrial plants. These waxes are hydrophobic and contribute to the plants barrier against the external environment protecting the plant from things such as water loss via evaporation.

II.C.4 Free Fatty Acids

Chemical characterization of the marigold extract identified a number of free fatty acids, which have not been further characterized for this GRAS application. However, within the scientific literature a number of authors have identified the particular fatty acids found in marigold flowers. Gong *et al.*, (2011) reported the dominant fatty acids of marigold flower after super critical CO_2 extraction to be linoleic, palmitic, and olenic acids. Lutein within marigold flowers may also be esterified to fatty acids, the predominant fatty acid is reported as palmitate which corresponds to the work conducted by Gong *et al.*, (2011). Other fatty acids that have been found esterified to lutein were myristate, dimyristate, stearate and distearate (Sujith et al., 2010). As these fatty acids have been found in both unextracted and extracted marigold flowers, it is

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considered appropriate to presume that they will also be present in the marigold extract currently under review. For this reason, the toxicological risk assessment in section VI.B addresses the food safety of these fatty acids.

II.C.5 Gums & Resinous Matter from Dried Marigold

Marigold extract contains a complex mixture of resinous and gum-like substances. A number of chemical analyses of the gums and resins found in marigold extracts are reported in the public literature, and are considered below.

II.C.5a Gums

Marigold flower extract is known to contain a large proportion of gum (Figueira et al., 1994). Plant gums are complex polysaccharides (other than starch) typically bound to protein, and are either cell wall components or provide a reserve energy store for the growth and development of the plant. Gums like pectin and gum acacia are widely used as additives in foods for their gelling and emulsifying properties. The gum extracted from marigold petals contains arabinose, galactose and glucose in the ratio 15:3:7 (Medina and BeMiller, 1993).

II.C.5b Resins

Resins, in the context of marigold flowers, are the high-molecular weight, hydrocarbon-rich portions of the hexane extracts. These hydrocarbons are primarily based on terpenes. Terpenes are derived biosynthetically from isoprene units (C_5H_8) which are linked together to form a vast variety of different molecules. Several authors have reported within the public domain on the constituents of marigold essential oils. The following essential oils were determined to be present using GC and GC/MS analysis: limonene (6.9%), terpinolene (4.7%), (z)-myroxide (7.9%), piperitone (28.5%), and caryophyllene (7.0%) (Krishna et al., 2004). Similarly, using GC and GC/MS, Arnas *et al.* (2012) identified high levels of piperitone (35.9%) and terpinolene (22.2%) in the oil. The yield of essential oils from plants is low; steam distillation extracted only 0.02-0.09% of essential oils from flowering marigold plants (Héthély et al., 1986). The essential oils that have been identified in marigold essential oil are commonly found in a variety of other botanical ingredients such as herbs. Given their low presence in marigold flowers, the concentration of the total or individual essential oils is expected to be low in the marigold extract.

II.C.6 Silicon dioxide (including sodium sulfate)

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(b) (4)

II.C.7 Vegetable oil

(b) (4)

II.C.8 Water

Water is present as a component of the silicon dioxide and the marigold plant.

II.C.9 Rosemary (in light of carnosic acid, phenolic diterpenes, flavonoids)

(b) (4)

II.C.10 Antioxidants

(b) (4)

II.C.11 Solvents

Solvent extraction is a process of diffusion of a solvent into oil-bearing cells of the raw material resulting in a solution of the oil in the solvent. The extraction process consists of treating the raw material with hexane and recovering the oil by distillation of the resulting solution of oil in hexane called miscella. Evaporation and condensation from the distillation of miscella recovers the hexane absorbed in the material. The extraction process of the dried marigold leaves in Cuatroxan is no different than described above and similar for almost all oil-bearing seeds or

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leaves. The manufacturer uses a quality plan, raw material risk assessment, risk justification and product specification to minimize any potential negative attributes for ingredient selection and finished goods manufacture.

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II.D Stability

II.D.1 Stability of Xanthophyll including Lutein in Marigold Extract

(b) (4)

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Table 6. Total xanthophyll content of Cuatroxan from original packaging at time 0 (six weeks post production) compared to C of A. Lot# 0608103730

Source ID	Xanthophyll Content g/kg
[redacted]	(b) (4)

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Table 7. Xanthophyll Content in Cuatroxan in Different Packaging Types

	Mean Xanthophyll Content (g/kg)				
	0.5 months	1 months	2 months	4 months	6 months
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(b) (4)					

Taken together, these data indicate that xanthophyll content (including lutein) in marigold extract (Cuatroxan) is stable for at least 6 months from the day of production.

II.D.2 Stability of Lutein in Pet Food

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(b) (4)

Table 8. Estimated Amount of Lutein^ in Pet Food at the End of Shelf Life

Diet	Type	Lutein remaining at the end of shelf life*
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Table 9. Stability of Lutein in Dog and Cat Food Stored at Elevated Temperatures

Diet	Type	% Lutein compared to Time 0	
		Day 28	Day 42
(b) (4)			

II.D.3 Homogeneity of Lutein in Pet Food

The homogeneity of marigold extract in pet food was assessed by measuring lutein from samples collected during the production runs of four different pet food recipes (Table 10). For each recipe, samples were collected every 15 minutes during the production run (total of 8 samples\ diet). The lutein concentration in each sample was measured and the coefficient of variance (CV) was calculated (Table 10).

The CV was below 15% for three of the recipes indicating that lutein is uniformly distributed throughout the pet food during the production run. In Dog Recipe B the CV was above 15% suggesting that lutein was not uniformly distributed. However, one of the values (sample 1) was confirmed using a Grubbs' test to be statistical outlier. When that value was removed, the CV for Dog Recipe B was below 15% indicating that lutein was uniformly distributed throughout the diet.

Taken together, these data demonstrate that lutein from marigold extract can be uniformly distributed in pet food.

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Table 10. Homogeneity of Lutein in Pet Food				
	Cat Recipe A	Cat Recipe B	Dog Recipe A	Dog Recipe B
%CV of 8 samples	4.4	12.1	11.9	24.4

II.E Analytical Methods

II.E.1 Method of Analysis for Xanthophyll and Lutein in Marigold Extract

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[redacted]

II.E.2 Method of Analysis for Lutein in Pet Food

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[redacted]

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III. HISTORICAL AND CURRENT REGULATED USE OF THE SUBSTANCE: Marigold Extract and Derivatives of Marigold Extract

III.A FDA Approved Uses

Marigold meal and extract from *T. erecta* (also known as Aztec Marigold) is approved as a color additive for use in chicken feed under 21 C.F.R. § 73.295 ["*Tagetes* (Aztec marigold) meal and extract"].[2] That regulation permits the use of marigold extract at levels consistent with good manufacturing practice (GMP) (*i.e.*, the minimum quantity required to produce its intended physical or technical effect, in this case coloring of the skin and eggs of chickens). The recommended application rate of marigold extract to achieve this effect varies by manufacturer. Cuatroxan (Kemin) is not intended for use in poultry feeds, but rather another marigold extract (Oro Glo 20, See Appendix F for product specification sheet) is used for this purpose. Kemin guarantees a xanthophyll activity content in Oro Glo 20 of not less than 20 g/kg (20,000 ppm, or 2%). Application rates vary with consideration of xanthophyll content from natural sources (corn, corn gluten meal, alfalfa meal, etc.) and/or any added red mixed oxycarotenoids in the ration. Therefore, while the amount added can be higher or lower, on a practical basis, typical use of the product is in the range of 0.5 to 1.0 pound per ton (500 ppm). The manufacturer of Nutri-Glo Yellow (Nutri-AD International) also guarantees a xanthophyll content of 20 g/kg, but recommends an application rate of up to four pounds per ton (2000 ppm), depending on content of carotenoids in the feed and required color (See Appendix B for product specification sheet). Based on analysis of Nutri-Glo Yellow by Royal Canin (it did not analyze the Oro Glo 20 product), the average lutein content is [redacted] (b) (4) (See Appendix D). Therefore, the final amount of added lutein to the chicken feed when Nutri-Gold Yellow is applied at the maximum rate recommended is 31.2 ppm.

The color additive regulation is not applicable to Royal Canin's intended use because the regulation is applicable only to chicken feed and because Royal Canin is using marigold extract for nutritive (antioxidant) purposes, rather than as a color additive. Royal Canin submitted its intention to use marigold extract as a nutritional source of lutein in pet food to the FDA Center for Food Safety and Applied Nutrition's (CFSAN) Office of Food Additive Safety, Division of Petition Review to ask whether Royal Canin would need to seek a color additive approval to cover that use. CFSAN confirmed that Royal Canin did not need to obtain a color additive approval to use marigold extract as a nutritional source of lutein in pet food (See Appendix C).

Preparations of marigold as an oil from *T. patula L.*, *T. erecta L.*, or *T. minuta L.* (*T. glandulifera Schrank*) is also approved for safe use in human food either as a natural flavoring substance or as

[2] *Tagetes* meal is defined as "the dried, ground flower petals of the Aztec marigold (*Tagetes erecta L.*) mixed with not more than 0.3 percent ethoxyquin," and the extract is defined as a "hexane extract of the flower petals of the Aztec marigold (*Tagetes erecta L.*)[,]... mixed with an edible vegetable oil, or with an edible vegetable oil and a hydrogenated edible vegetable oil, and not more than 0.3 percent ethoxyquin [that]... may also be mixed with soy flour or corn meal as a carrier." Ethoxyquin is used as an antioxidant/preservative to retard oxidation of the carotenoids.

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a substance used in conjunction with flavoring substances under 21 C.F.R. § 172.510 ("Natural flavoring substances and natural substances used in conjunction with flavors"). The additive is permitted at levels consistent with GMP. The substances listed in this part, including marigold oil as described above, are also permitted for use in animal feed, including dog and cat food, as a "natural flavor" under 21 C.F.R. § 501.22(a)(3).

III.B GRAS Notifications

Lutein in various forms has been the subject of nine GRAS Notices for use in human food applications, *i.e.*, GRAS Notices 110 (Lutein esters), 140 (Crystalline lutein), 221 (Suspended lutein), 291 (Crystalline Lutein), 385 (Lutein), 390 (Suspended lutein), and 432 (Lutein diacetate), 542 (Lutein) and 543 (Lutein esters) (GRAS Notice Inventory Listings at http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing). The FDA has had no objections to any of these Notices. In response to separate GRAS notices on lutein, the FDA responded that they had no questions regarding the safety of lutein as described in those documents. The use of lutein in specific foods in these notices was estimated to result in levels of up to 13.4 mg/day and was considered to be safe (GRN 385, 2011).

GRAS Notice 110 regarded a mixture of carotenoid esters derived from marigold flowers (*Tagetes erecta)* for use in a variety of conventional human foods and medical foods, intended as the sole active item of the diet, in levels not to exceed 40 mg/d (GRN 110, 2003). GRAS Notice 140 regards the use of crystalline lutein, sold as FloraGLO®, also for the use of lutein as an ingredient in medical foods intended as the sole item of the diet, in levels not to exceed 20 mg/day (GRN 140, 2004). GRAS Notice 221 regards the use of suspended lutein, sold as FloraGLO® Lutein 20% in Safflower Oil, as a component in term infant formula at a maximum level of 250 micrograms/liter (GRN 221, 2007). Crystalline lutein, sold as FloraGLO® has also been determined to be GRAS for use as an ingredient in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, chewing gum, dairy product analogs, fats and oils, frozen dairy desserts and mixes, gravies and sauces, hard candy, infant and toddler foods (at levels of up to 1 mg/serving other than for infant formula), milk products, processed fruits, vegetable products, and soft candy at levels ranging from 0.3 to 3.0 milligrams per RACC (GRN 291, 2009). GRAS Notice 385, for lutein, also regards the use of lutein as an ingredient in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, chewing gum, dairy product analogs, egg products, fats and support oils, frozen dairy desserts and mixes, gravies and sauces, hard candy, infant and toddler foods (other than infant formula), milk products, processed fruit and fruit juices, soft candy, soups and soup mixes, and medical foods intended as the sole item of the diet, at levels ranging from 0.3 to 3.0 milligrams (mg) per RACC. The estimated dietary intake for 90th percentile users was estimated to be 13.4 mg/p/d or 0.22 mg/kg.bw/day from the covered uses (GRN 385, 2011). GRAS Notice 390, for suspended lutein, regards the use of lutein as an ingredient in formulas intended for premature infants at levels up to 210 micrograms of lutein per liter of formula (GRN 390, 2012). Lastly, GRAS Notice 432 regards the use of lutein diacetate, Notice 542 regards the use of lutein, and Notice 543 regards the use of lutein esters, in each case as an ingredient in baked goods and baking mixes, beverages and beverage bases, breakfast cereals, chewing gum, dairy product analogs, fats and oils, frozen dairy desserts and mixes, gravies and sauces, hard candy, infant and toddler foods (excluding infant formula), milk products, processed fruits and fruit juices, soft candy, and soups and soup mixes (and, in the case of lutein and lutein esters, egg substitute products) at

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levels of 0.3 to 3 milligram per serving (GRN 432, 2012; GRN 542, 2015; GRN 543, 2015). Overall, the FDA has determined lutein to be GRAS for use as an ingredient in human food, including use as an ingredient in infant formula.

III.C Other Organizations

The NRC of the National Academies was commissioned by the FDA to convene a committee of experts to report on factors affecting the safe use of dietary supplements in various domestic animal species. The report, entitled *Safety of Dietary Supplements in Horses, Dogs and Cats*, was published in 2009. As part of the report, the committee included a complete assessment of lutein in these species. Based on studies showing no apparent adverse effects at levels used, the report offers a presumed safe intake of 1.8 mg/kg.bw/day for dogs and 7.2 mg/kg.bw/day for cats (National Research Council, 2009).

Lutein from *Tagetes erecta* was evaluated by the Joint Expert Committee on Food Additives (JECFA) in 2004 (JECFA, 2004). The level at which acute toxicity was seen in rats was determined to be greater than 2000 mg/kg.bw/day. Based on several sub-chronic studies, including developmental toxicity, no adverse effects were documented in laboratory animals (*i.e.*, mice, rats, monkeys) or clinical trials in humans. Further, in a number of studies reviewed by JECFA, no evidence of genotoxicity was found. Based on these data, and considering that lutein was not genotoxic, had no structural alerts for carcinogenicity, is a natural component of the diet, and also is normally found in many sites within the body, especially the eye, JECFA did not consider a chronic study necessary.

Lutein is registered as an additive for human in food in the EU at concentrations up to a maximum of 100 mg/kg in a number of processed foods (European Commission, 2008).

In Europe, lutein is registered under feed regulations as an authorized food additive (colorant) for poultry at a maximum concentration of 80 mg/kg of complete feeding stuffs (European Commission, 2003). Lutein is not listed as a material whose placing on the market or use for animal nutritional purposes is restricted or prohibited as regulated by Regulation (EC) No 767/2009 (European Commission, 2009), nor is it considered an undesirable substance as regulated by Directive 2002/32/EC (European Commission, 2002).

IV. INTENDED USE OF THE SUBSTANCE: Marigold Extract

IV.A Proposed Use of Marigold Extract in Dog and Cat Foods

Marigold extract contains a high level of the nutritional antioxidant lutein (See Section II.C). The lutein in marigold extract has a number of benefits for cats and dogs when consumed in their diet (See Section V). For these reasons, Royal Canin proposes to add marigold extract to dog and cat foods in sufficient amounts to supply no more than 5 mg lutein per 1000 kcal ME (Table 11). The exact amount of marigold extract used will depend on the energy density of the product and the concentration of lutein in the commercial extract. A diet intended for dogs with high energy needs (e.g., working dogs) or for cats may require use of an extract with a higher concentration of lutein, which can be added in lower amounts. (b) (4)

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Table 11: Intended Use		
Product	Maintenance diet	High energy diet
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IV.B Estimated Dietary Intake (EDI) Resulting from Proposed Use

IV.B.1 Adult Dogs

At the inclusion rates for the two examples of dog food reported above, dogs at maintenance (kcal per day = 130 X $kg.bw^{0.75}$) or at twice-maintenance ("High energy") requirements as needed for work (kcal per day = 260 X $kg.bw^{0.75}$) will consume between 0.23 and 1.27 mg lutein/kg.bw/day (Table 12).

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Table 12: Inclusion Rate								
Dog Weight [kg]	Energy requirements [kcal/day]		Feed intake [g/day]		Lutein intake [mg/day]		Lutein intake [mg/kg.bw/day]	
	Maintenance	High energy	Maintenance	High energy	Maintenance	High energy	Maintenance	High energy

(b) (4)

IV.B.2 Puppies

Puppies will consume more lutein per kilogram body weight than will adult dogs at maintenance. According to NRC (National Research Council, 2006), a 16-week-old, 17-kg Labrador puppy with an anticipated adult size of 35 kg requires 1934 kcal ME per day. A younger, 1.5-kg small-breed puppy with an anticipated adult size of 5.0 kg requires 308 kcal ME per day. At a maximum inclusion rate of 5 mg lutein per 1000 kcal ME, the larger puppy would consume 9.67 mg lutein (0.57 mg/kg.bw) per day, while the smaller puppy would consume 1.89 mg lutein (1.26 mg/kg.bw) per day.

IV.B.3 Cats and Kittens

Kittens will consume more food per kilogram body weight than will adult cats. According to NRC (National Research Council, 2006), a 0.8-kg kitten with an anticipated adult weight of 4 kg requires 175 kcal ME per day. Therefore, at a maximum inclusion rate of 5 mg lutein per 1000 kcal ME, it would consume 0.875 mg lutein (1.09 mg/kg.bw) per day.

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V. CONDITIONS OF USE AND UTILITY: Marigold Extract

V.A Background

Carotenoids are ubiquitous in plant-based foods where they provide the yellow, orange, and red pigmentation of fruits and vegetables. Approximately 600 carotenoids have been identified and can be classified into two different types, carotenes and xanthophylls. The key dietary carotenes, β- carotene and lycopene, are responsible for the orange and red pigments, respectively, in carrots, cantaloupe, and tomatoes. Xanthopylls, such as lutein and zeaxanthin, provide the yellow pigmentation in corn and orange peppers.

Marigold petals, like many plants, contain high levels of the carotenoids, particularly the yellow pigment lutein (Section II.C). For this reason the extract from marigold petals has historically been used as a color additive in chicken feed and a natural flavor for human food and animal feed. In the United States, preparation of marigold as an oil from *T. patula L.*, *T. erecta L.*, or *T. minuta L.* (*T. glandulifera Schrank*) is approved for safe use in human food either as a natural flavoring substance or as a substance used in conjunction with flavoring substances under 21 C.F.R. § 172.510. These substances are also permitted for use in animal feed, including dog and cat food, as a "natural flavor" under 21 C.F.R. § 501.22(a)(3).

Although coloring is an important property, carotenoids also have a critical cellular function as antioxidants. Structurally they consist of long chains of hydrocarbons that enable the quenching of reactive oxygen species (Stahl and Sies, 2003). Unlike carotenes, xanthophylls, like lutein, have hydroxyl groups on each end (Table 4) making them stronger antioxidants compared to other carotenoids (Baskin et al., 2000). In fact, lutein is five times more effective as an antioxidant than is retinol (Jaramillo-Flores et al., 2003).

Lutein possesses potent antioxidant activity, including the ability to scavenge superoxide radicals, hydroxyl radicals, and inhibit lipid peroxidation (Sindhu et al., 2010). Furthermore, oral administration of lutein in mice significantly inhibited superoxide generation (Sindhu et al., 2010). Because of this strong antioxidant activity, lutein derived from marigold extract has been the subject of many GRAS Notifications for use in human food, i.e., GRAS Notices 110 (Lutein esters), 140 (Crystalline lutein), 221 (Suspended lutein), 291 (Crystalline Lutein), 385 (Lutein), 390 (Suspended lutein), and 432 (Lutein diacetate), 542 (Lutein) and 543 (Lutein esters) (GRAS Notice Inventory Listings at http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing). The FDA has had no objections to any of these Notices and in each case has responded that they had no questions regarding the safety of lutein as described in those documents.

While these Notifications as submitted to CFSAN do not explicitly identify or substantiate proposed utility of the substance, it is reasonable to presume that lutein is added to these human foods for its antioxidant value, not as a flavor, color, or for technical purposes. Similarly, Royal Canin's intended use of marigold extract would be for its nutritive value as a source of the potent antioxidant, lutein.

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V.B Antioxidant Activity of Dietary Carotenoids

The antioxidant properties of lutein have been demonstrated (Sindhu et al., 2010) and the potential benefits for maintenance of visual function and health have been reviewed (Granado et al., 2003). Carotenoids, including lutein, are a critical part of the defense mechanisms to control cellular damage resulting from oxidation.

Oxidation is critical for proper cellular function in plants and animals; however it results in the production of reactive oxygen species, including singlet oxygen, hydroxyl radicals, superoxides and peroxides. Accumulation of these reactive oxygen species results in DNA damage, lipid peroxidation, and cell death. For this reason dietary carotenoids, through their antioxidant properties, play an important physiological role in helping maintain normal structure and function of the animal's body. For instance, a study has shown that dietary antioxidants, including lutein, decrease the DNA damage and increase the resistance of lipoprotein oxidation during prolonged exercise in sled dogs (Baskin et al., 2000).

Oxidative stress or reactive oxygen species accumulation can lead to significant DNA damage, including impaired base pairing, DNA replication or transcription, base loss, or single stranded breaks (reviewed by Jackson and Bartek, 2009). The antioxidant activity of the carotenoids lycopene, astaxanthin, zeaxanthin, and lutein has been reported to protect against DNA damage (reviewed by Azqueta and Collins, 2012). In addition dietary antioxidants have been shown to improve mitochondrial function in dogs, and the authors suggest this is a result of alleviating oxidative damage to cellular DNA (Park et al., 2013). In another study dietary antioxidants, including lutein, were reported to exert a protective effect by significantly decreasing DNA damage compared to dogs feed diets without antioxidants (Heaton et al., 2002).

The retina generally, and the macula and fovea of humans and nonhuman primates in particular, have naturally elevated levels of lutein. It is perhaps not surprising that these antioxidant compounds spontaneously accumulate at this location given the retina's high metabolic activity and the photo-oxidative stress to which it is continually exposed during waking hours. In plants, carotenoids are essential for controlling the photo-oxidative stress that occurs during photosynthesis. Like in plants, carotenoids such as lutein and zeaxanthin are believed to help maintain ocular health by acting as antioxidants and/or by elevating macular pigment concentrations, thereby enhancing resistance to potentially damaging effects of short-wavelength light (Neuringer et al., 2004; Johnson et al., 2008). As a result, the safety and utility of antioxidants in general, and lutein in particular, as nutrients important for support of ocular health in humans and nonhuman animals have received much research attention (For recent reviews see Williams, 2008; Grover and Samson, 2014). In particular these compounds are sometimes recommended for dietary supplementation of people (Wu et al., 2015).

To date, such *in vivo* work has been conducted largely in humans (clinical trials) or in nonhuman primates (biochemical, pharmacokinetic, and experimental models) because other mammals lack the macula or fovea where lutein and zeaxanthin naturally accumulate. However, a recent study demonstrated that dogs have a macula-like region within their retina (Beltran et al., 2014) suggesting lutein is an interesting compound for study of its potential role in maintenance of structure and function of the canine eye. In summary, data from experimental models and from

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larger epidemiological studies in clinically relevant situations broadly support a role for lutein and zeaxanthin in maintaining ocular heath.

In addition to ocular health, dietary carotenoids have been shown to promote a healthy immune response via their function as antioxidants, including immunoglobulin production, lymphoblastogenesis, lymphocyte cytotoxic activity, cytokine production, and delayed type hypersensitivity (reviewed by Chew and Park, 2004). In fact, dietary antioxidant cocktails that include lutein have been shown to increase the speed at which dogs and cats achieve protective immunity following vaccination (Heaton et al., 2002).

Although many of these studies have focused on the immune effects of a blend of antioxidants or dietary β-carotene, lutein has also been shown to affect humoral and cellular immune response in cats and dogs (Kim et al., 2000b, a). Cats fed diets containing varying amounts of lutein had a significant dose-related increase in delayed type hypersensitivity response to vaccines, significantly higher Th and B cell subpopulations, and increased plasma IgG concentrations (Kim et al., 2000a). Similarly, dogs fed varying amounts of lutein had significantly increased delayed type hypersensitivity response, increased CD4+Th cells and IgG production (Kim et al., 2000b). These data indicate that dietary lutein promotes normal cell-mediated and humoral immune responses in cats and dogs (Kim et al., 2000b, a).

V.C Fate of Dietary Lutein

Lutein is a widely-distributed carotenoid produced by chloroplastic plants, thus it is among the most prevalent carotenoids in the North American diet (IOM, 2000). Lutein is found in high concentrations in green leafy vegetables, such as spinach and kale, broccoli, Brussel sprouts, cabbage, and green beans (Khachik et al., 1995) (Section VI.A.1 Table 13). The average American consumes between 1 and 2 milligrams of lutein per day although a safe level of 4-8 milligrams has been established by the USDA (USDA, 2015). Marigold flowers also contain a significant amount of lutein (Section II.C, Table 2) and for this reason lutein is the subject of this GRAS notification.

Due to the absence of appropriate endogenous enzymes, animals cannot synthesize lutein *de novo* or convert it from immediate precursors in the diet. Numerous studies have confirmed that ingestion of lutein in the diet increases plasma antioxidant levels and activity in a dose dependent manner in dogs and cats (Baskin et al., 2000; Kim et al., 2000b, a; Heaton et al., 2002). Lutein is absorbed by the gut in a manner similar to dietary fat and then distributed to tissues via transport by lipoproteins.

Animals appear to readily utilize absorbed lutein, suggesting an important role in normal nutrition. In humans, lutein preferentially accumulates in the retina where it provides key benefits for ocular health. Although dogs and cats lack a true macula or fovea where lutein naturally accumulates, dogs do have a macula-like region within their retina (Beltran et al., 2014), suggesting lutein could also play an important role in maintenance of proper eye function in this species.

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Lutein also accumulates in the lungs, spleen, red blood cells, and other tissues, but not in the brain or in fat (National Research Council, 2009). Detection of isomers and esters of lutein in assorted tissues suggest a complex metabolism after absorption and transport, akin to the metabolism of a nutrient versus simple degradation and/or excretion of a xenobiotic (e.g., a drug).

V.D Studies with lutein as a nutritive antioxidant that supports the normal development of dogs and cats

V.D.1 Introduction

As described in previous sections, Marigold extract contains a significant amount of lutein, and, because of the lutein derived-yellow pigmentation, has been commonly used as a food and feed colorant. In addition to lutein's pigmentation qualities, researchers have begun to focus on its antioxidant properties. Lutein is a natural, fat soluble antioxidant and has been identified as a potent scavenger of free radicals (Sindhu et al., 2010). Functioning as an antioxidant, lutein can help prevent damage to the immune system, as demonstrated by maintenance of a healthy immune response. Several studies have recently shown that dietary lutein has nutritive benefits in cats and dogs, namely supporting the immune response and reduction of exercise-induced oxidative damage.

V.D.2 Kim et al., Modulation of humoral and cell-mediated immune responses by dietary lutein in cats (Kim et al., 2000a)

Objective

To investigate the effects of dietary lutein on humoral and cell-mediated immune responses in female tabby cats.

Methods

Fifty six animals received basal diets supplemented with 0, 1.5, or 10 mg lutein per day for 12 weeks. These doses corresponded to 0.71, 3.54, or 7.09 mg lutein/kg.bw/day, and 0.05, 0.24, or 0.48 mg zeaxanthin/kg.bw/day, respectively. Throughout the study, body weights were recorded and blood samples were collected for analysis of delayed type hypersensitivity (DTH) response, mitogen-induced peripheral blood mononuclear cell (PBMC) proliferation, changes in PBMC sub-populations, interleukin-2 (IL-2) production, and plasma immunoglobulin (IgG) concentrations. In addition, DTH response to concanavalin A (Con A) or a polyvalent vaccine was assessed on weeks 0, 6, and 12.

Results

Lutein, when added to the diet of cats, resulted in dose-dependent increases in plasma lutein concentration. A significant dose-related increase in DTH response to vaccine but not to Con A,

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and significantly enhanced Con A- and pokeweed mitogen (PWM)-stimulated PBMC proliferation was reported.

Conclusions

The antioxidant lutein, when consumed in the diet, supports cell-mediated and humoral immune responses in domestic cats.

V.D.3 Kim et al., Dietary lutein stimulates immune response in the canine (Kim et al., 2000b)

Objective

To investigate the effects of dietary lutein on the immune response in dogs.

Methods

Fifty-six dogs received basal diets supplemented with 0, 5, 10, or 20 mg lutein day for 12 weeks corresponding to 0.44, 0.88, or 1.75 mg lutein/kg.bw/day and 0.03, 0.06, or 0.12 mg zeaxanthin/kg.bw/day. Throughout the study, body weights were recorded and blood samples were collected for analysis of lymphocyte proliferative response to phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM), changes in peripheral blood mononuclear cell (PBMC) populations, IL-2 production, and IgG and IgM production.

Results

Lutein, when added to the diet of dogs, resulted in dose-dependent increases in plasma lutein concentration. Lutein supplementation significantly increased the DTH response to vaccine and phytohemagglutinin (PHA) and significantly increased mitogen- (PHA, Con A, and PWM) stimulated PBMC proliferation. The percentages of cells expressing CD5, CD4, CD8 and major histocompatibility complex class II molecules were significantly increased, and the production of IgG in lutein-supplemented animals significantly increased after the second antigenic challenge. There were no differences in IL-2 production, plasma IgM, or expression of the CD21 lymphocyte marker throughout the experimental period.

Conclusions

The antioxidant lutein, when consumed in the diet, supported cell-mediated and humoral immune responses.

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V.D.4 Baskin et al., Effects of dietary antioxidant supplementation on oxidative damage and resistance to oxidative damage during prolonged exercise in sled dogs (Baskin et al., 2000)

Objective

To investigate the effects of antioxidant supplementation, including lutein, on indices of exercise-induced oxidative damage in Alaskan sled dogs.

Methods

Sixty-two dogs were assigned to one of three treatment groups: 1) sedentary and nonsupplemented (group C); 2) exercised and supplemented (group S) and; 3) exercised and nonsupplemented (group N). All groups were fed the same basal diet and the supplemented group received 400 units of alpha-tocopherol acetate, 3 mg of beta carotene, and 20 mg of lutein orally for 30 days. Groups S and N were then subjected to three days of exercise. Blood samples were collected from all groups before the start of exercise and after Days 1 and 3 of the exercise program. Plasma antioxidant concentrations were measured in all groups. Oxidative damage to DNA was monitored via plasma 7,8 dihydro-8-oxo-2'deoxyguanosine (8-oxodG) concentration and plasma hydroperoxide concentration, as well as resistance of plasma lipoproteins to oxidation.

Results

Supplementation increased plasma tocopherol levels by 72% compared to the control and unsupplemented groups. Beta-carotene concentrations averaged 0.003 micromol/L in the supplemented group but were not detectable in the groups not receiving beta-carotene supplementation. Lutein supplementation increased plasma concentrations by 91%. In group S, plasma concentration of 8-oxodG decreased significantly during and after exercise. Conversely, group N exhibited significant increases in 8-oxodG during and after exercise. Exercise resulted in an increase in the lag time of lipoprotein oxidation in group S but no increase in group N. There was no change in lipoprotein oxidation lag time in Group C. Supplementation or exercise had no effect on plasma concentration of hydroperoxides. A decrease in plasma lutein and an increase in plasma tocopherols during exercise were observed in the supplemented group. Group N exhibited a decrease in plasma tocopherols after day one of exercise and no change in plasma lutein.

Conclusions

Dietary supplementation with antioxidants including lutein decreased DNA damage and increased the resistance of lipoproteins to oxidation during exercise in dogs.

V.D.5 Discussion and Conclusions

These studies provide evidence that dietary lutein has a powerful nutritional benefit to dogs and cats. They have demonstrated that dietary lutein is absorbed into the plasma and that increased

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concentration of plasma lutein protects cells from oxidative damage, which further supports the immune response in dogs and cats.

In each of these studies lutein was provided in the diet with other carotenoids or antioxidants. For instance, in the studies investigating the effects of lutein on immune response in dogs in cats, the diets were supplemented with lutein and zeaxanthin (Kim et al., 2000b, a). The lutein used was derived from marigold flower petals, and therefore was similar to marigold extract (the subject of this GRAS notification) in that it contained both lutein and zeaxanthin (see Section II.C). In the study investigating the effects of antioxidant supplementation on oxidative damage in sled dogs, lutein, α-tocopherol acetate, and β-carotene were supplemented in the diets (Baskin et al., 2000). Although these three antioxidants were used, the authors conclude that lutein may be preferentially utilized to prevent oxidative stress because plasma concentration of lutein decreased after the exercise period and throughout the rest period.

Overall these studies demonstrate that dietary lutein derived from marigold is absorbed into the plasma where it confers nutritive antioxidant benefits to dogs and cats.

VI. DOCUMENTATION TO SUPPORT THE SAFETY OF THE SUBSTANCE: Marigold Extract

Marigold extract is derived from the flowers of *Tagetes erecta*. Although not commonly consumed in North American, Marigold petals have a long history of safe use in human diets. In 1917 "the Herbalist" indicated that Marigold petals were a source of vitamin C and phosphorus (Smith, 1973). Because of their color and nutritive benefits, marigold petals have been used in a variety of culinary applications including in salads and with eggs (Smith, 1973). The petals are often substituted in place of saffron and tarragon in casseroles, vegetable dishes, and in venison and seafood recipes (Smith, 1973). In South America, marigold petals are made into a paste and are a main component of a potato dish called ocopa. Additionally because of their nutritional value, the petals have been routinely fed to farm animals, such as pigs and chickens. Marigold petals from which marigold extract is derived have a long history of safe use in both human and animal diets.

Marigold extract contains a significant amount of the antioxidant lutein (See Section II.C). Dietary lutein has a number of nutritive benefits for cats and dogs (See Section IV), and it is for this reason that it is the subject of this GRAS notification.

VI.A Natural Occurrence of Lutein in the Diet

VI.A.1 Occurrence of Lutein in Human Food Items

Lutein and zeaxanthin are among the most prevalent carotenoids in the North American diet (IOM, 2000). Both carotenoids are found in high concentrations in green leafy vegetables, such as spinach and kale (Khachik et al., 1995; Omaye et al., 1997), and in chicken egg yolks (292 and 213μg/egg yolk of lutein and zeaxanthin, respectively) (Handelman et al., 1999). Lutein is also abundant in broccoli, Brussel sprouts, cabbage, and green beans (Khachik et al., 1995). In contrast, zeaxanthin is only present at modest concentrations in corn (Khachik et al., 1995) but at

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much higher concentrations in corn gluten (Bodwell and Nelson, 1990; Moros et al., 2002). Luteins and other carotenoids are also found as dietary supplements. The average American consumes between 1 and 2 milligrams of lutein per day although a safe level of 4-8 milligrams has been established by the USDA (USDA, 2015). Table 13 shows naturally occurring levels of lutein and zeaxanthin in various vegetables.

Table 13: Lutein and Zeaxanthin Concentration in Fruits and Vegetables (Torrey, AMDF)

Nutritional database	Food	Lutein and zeaxanthin content (mcg)		Serving size (Cup)
		Per 100g	Per serving	
11233	Kale, raw	39,550	22,148	1
11234	Kale, drained Cooked, boiled	15,798	16,903.86	½
11569	Turnip Greens, drained, cooked	8,440	9,030.8	½
11162	Collards,drained cooked	8,091	8,657.37	½
11461	Spinach, drained cooked	7,043	7,536.01	½
11457	Spinach, raw	11,938	6,685.28	1
11091	Broccoli,drained cooked,	2,226	1,736.28	½
11168	Corn, drained sweet, yellow, cooked,	1,800	1,476	½
11251	Lettuce, raw	2,635	1,475.6	1
11308	Peas, drained solids green, canned	1,350	1,147.5	½
11099	Brussels Sprouts, drained, cooked	1,290	1,006.2	½
11172	Corn, drained sweet, yellow, canned, whole kernel	884	724.88	½

VI.A.2 Occurrence of Lutein in Pet Foods

The most common sources of lutein in pet food diets are corn products and dried egg. Due to their intrinsically high lutein content, dried spinach and corn gluten meal also have been used

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specifically to fortify dietary lutein levels. Analysis conducted by Kemin Industries on samples submitted by a pet food manufacturer found the following lutein levels in dried vegetables offered for pet food applications (Table 14).

Table 14: Lutein Content of Common Pet Food Ingredients

Sample	Lutein level (ppm)
Dried Tomato	**7.9**
Dried Carrot	**6.4**
Dried Broccoli	**52.9**
Dried Spinach	**322.3**
Corn	**6.0**
Corn Gluten	**154.0**
Dry Batch	**35.0**
Dry Extruded Kibble	**30.8**

Research conducted at the University of Guelph (Leeson and Caston, 2004) assessed increased lutein levels in egg yolks as an enriched dietary nutrient source. In these experiments, chicken corn/soy diets were directly supplemented with up to 1000 ppm lutein. A significant increase in yolk lutein from a basal level of 0.3 to 1.5mg/60g egg was observed.

However, supplementation levels > 375ppm resulted in no significant increase in yolk lutein above 1.5 mg/60 g ($P > 0.05$). Adding corn gluten and alfalfa to the chicken's diet increased yolk lutein to 2.2mg/60g egg with that content leveling off at 500 ppm dietary lutein. Based on the basal corn/soy diet, a fresh egg typically contains 0.3 mg/60g or 5mg/kg (5 ppm). Converted from a 37.7% whole egg moisture content to a dried basis (10% moisture) and assuming no loss through spray drying, the typical lutein content of dried egg is 7.2 ppm (Table 15). Enrichment through added lutein or corn gluten/alfalfa increased yolk lutein to a maximum of 7X the basal (or 53 ppm) lutein for dried egg.

Table 15: Lutein Content of Whole & Dried Egg Produced by Chickens Fed Basal & Lutein-Enriched Diets

Chicken Diet	Max. Lutein Level (Whole Egg)	Max. Lutein Level (Dried Egg)
Egg Moisture	**37.7%**	**10%**
Basal Corn/Soy	**0.3ma/60g egg**	**7.22 ppm**
Corn/Soy + Lutein	**1.5ma/60g egg**	**36.12 ppm**
Corn/Soy + Corn Gluten & Alfalfa	**2.2mg/60g egg**	**53.00 ppm**

Data from Leeson, 2004

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(b) (4)

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Table 16: Correlation of Post-Production Dry Pet Food Diet Lutein Content to Corn Gluten Meal Position on the Ingredient Panel				
Dry Diet	**Predicted Lutein**	**Zeaxanthin (ppm)**	**Lutein (ppm)**	**Corn Gluten Position**
(b) (4)				

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(b) (4)

Table 17: Expected Lutein Content for Dry and Canned Pet Foods

Diet Type	"As Fed" Moisture (%)	"As Fed" Lutein (ppm)	Dry Basis Lutein (ppm)
(b) (4)			

Addition of marigold extract at Royal Canin's proposed use levels of no more than 5 mg per 1000 kcal ME will add approximately 15 to 25 ppm lutein to the diet. When added to a food that is inherently low in lutein content, it can raise the lutein content to levels comparable to those formulated with corn gluten meal or other high-lutein ingredients.

VI.A.3 Estimated Daily Intake of Lutein in Dogs and Cats from Commercial Pet Foods

The daily caloric requirements of dogs can vary greatly depending on breed and body type. The energy requirements for the average laboratory kennel dogs and active pet dogs at maintenance can be estimated by the formula; kcal ME per day = 130 X $kg.bw^{0.75}$ (National Research Council, 2006). Based upon the range of lutein contents in commercial pet foods as discussed above, and using an average energy content of pet foods (4000 kcal ME/kg dry

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matter), the estimated intake of lutein in a small (5 kg) adult dog ranges from 0.06 mg/kg.bw/day for a diet low in lutein (3 ppm DM) to 1.09 mg/kg.bw/day for a diet high in lutein (50 ppm DM). Thus, the estimated intake of lutein in a large (50 kg) adult dog ranges from 0.04 to 0.61 mg/kg.bw/day for the low and high-lutein diets, respectively.

Cats do not exhibit as large of a range in caloric requirement as dogs. The energy requirements for an adult cat at maintenance in lean body condition can be estimated by the formula kcal ME per day = 100 X $kg.bw^{0.67}$ (National Research Council, 2006). A 4.5 kg adult, non-reproducing cat eating a food containing 4000 kcal ME/kg dry matter would consume 0.05 mg/kg.bw/day when fed a diet containing 3 ppm lutein DM, or 0.76 mg/kg.bw/day when fed a diet containing 50 ppm lutein DM.

VI.B. Toxicological Risk Assessment in Dogs and Cats

As stated previously, this GRAS specifically addresses marigold extract and not the additional ingredients. However the extraction procedure for marigold remains similar and therefore the relative composition of the chemicals within the marigold extract compared to the lutein fraction are not expected to change significantly.

As Royal Canin intends to add lutein at no more than 5 mg/1000kcal ME and assuming a (b) (4) Given the relative contributions of the other components within the marigold extract to lutein, the dietary exposure has been worked out for puppies, again as a worst case scenario. In the interests of transparency this has also been conducted for the wider ingredients within the Cuatroxan product. It should be noted that if other formulations of marigold extract are used, these toxicological risk assessments for the other components would not be necessary.

A risk assessment follows for each identified fraction within the Cuatroxan.

VI.B.1 Lutein and Zeaxanthin

There are insufficient toxicological studies available to base a risk assessment on the marigold extract as characterized by the flow diagram in Table 1. Therefore the toxicity of marigold has been considered in light of the natural botanicals present in the material as identified in Table 2.

Lutein and zeaxanthin are isomers with similar structural, physiological and toxicological properties. In light of these similarities, for the purposes of this toxicological risk assessment they have been grouped together in line with the JECFA evaluation (JECFA, 2006).

Lutein has been evaluated separately by a number of international and regulatory bodies including JECFA, EFSA, and the NRC on behalf of the FDA. Zeaxanthin has also been evaluated separately by EFSA and JECFA. The below section summarizes the information from these reviews, with only specific studies considered pivotal to the risk assessment mentioned.

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Results of additional studies reported after the publication of these evaluations are also included below.

Acute Toxicity
Based on reported LD_{50}s in rats of greater than 2000 mg/kg.bw, lutein is of low acute oral toxicity (reported in JECFA, 2006).

Irritation and Sensitization
Crystalline lutein is not considered irritating to rabbit skin after a skin irritation study in New Zealand White Rabbits. Crystalline lutein was also not considered irritating to the eye in the same species (reported in JECFA, 2006).

Repeat Dose
A number of studies are reported by JECFA and EFSA in which no adverse effects were attributable from treatment with lutein. The pivotal study identified by both JECFA and EFSA was a 13-week study in rats. In this study conducted according to OECD Test Guideline 408, Han Wistar rats (10/sex/dose) were administered a lutein extract from marigold flowers (lutein content: 79%) in their diets at doses of 0, 2.6, 26, or 260 mg/kg.bw/day (reported to be 0, 2, 20 or 200 mg/kg.bw/day, respectively, when accounting for the lutein content of 79%). At the end of the study period, all animals were sacrificed and necropsied except for 5 animals from the control group and 5 from the highest dose group which were allowed a recovery period of 4 weeks before necropsy. The authors reported no adverse clinical signs and all animals except one survived the duration of the study; the animal death was attributed to blood sampling and not treatment related. There were no changes in hematology or clinical chemistry values, and histopathology did not reveal any changes attributable to the treatment. JECFA and EFSA identified a No Observed Effect Level (NOEL) based on the highest dose of the study of 260 mg/kg.bw/day, which when lutein content in the extract (79%) was accounted for, was calculated to be 200 mg/kg.bw/day (JECFA, 2006; EFSA, 2010b).

Carcinogenicity
No lifetime or carcinogenicity studies are reported in the literature.

Genotoxicity
Lutein has been evaluated in a number of *in vitro* and *in vivo* assays including bacterial reverse mutation assay, chromosomal aberration assay, *in vivo* micronucleus assay and comet assay (EFSA, 2010b). All results were negative leading EFSA and JECFA to conclude that lutein is not considered genotoxic (JECFA, 2006; EFSA, 2010b).

Reproductive and Developmental Studies
No multi-generation studies are reported for lutein. In a developmental study in Sprague Dawley rats, females were administered in their diet beadlets containing 10% lutein (from marigold extract: 79% lutein, 5% zeaxanthin) on days 6 to 20 of gestation. Diets were formulated to contain 250, 500 or 1000 mg/kg.bw/day (based on actual intake, this was estimated to be 252, 535 or 1118 mg/kg.bw/day). There was no evidence of adverse effects to the dams; although food intake was decreased at the lower doses, this was attributed to reduced palatability of the placebo beadlets (without lutein). Decreased food intake resulted in lower maternal and fetal

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bodyweights and delayed ossification in animals fed the lower doses. However, there were no adverse effects on, pre- or post-implantations losses, fetal survival, sex ratios or the incidence of abnormalities. The study authors identified a NOEL of 1000 mg/kg.bw/day, the highest dose in the study (JECFA, 2006; EFSA, 2010b).

In a two-generation reproduction toxicity study conducted according to OECD Guideline 416 in Crl:WI(Glx/BRL/HAN)BR rats (6/sex/dose). Rats were fed synthetic zeaxanthin formulated into beadlets that were mixed into diets at doses of 0, 52, 155 or 508 mg/kg.bw/day, with the control group receiving placebo beadlets. As per OECD Guideline 416, parents were fed the supplemented diet for approximately 10 weeks before mating, dosed throughout the two-week mating period, and females were dosed throughout the gestational period. The F1 and F2 generation were therefore exposed, neonatally (via breast milk) and from weaning (via access to the same diets as their parents). All surviving animals were sacrificed and necropsied at the end of the study period. In the parental generation administration of zeaxanthin did not show any adverse effects; there were no changes in body weight, body weight gain, food intake, estrous cycling, mating, fertility, fecundity, gestational period, number of implantations, number of pups, sex ratio, pup survival or bodyweight development. A number of females aborted; however these were not dose-dependent and also included control animals and therefore was not considered related to the treatment. At the highest dose, there was a lower post-implantation survival, however this was not statistically analyzed. Necropsy of the parents did not show any adverse effects related to treatment. In the F1 generation, the authors reported that two animals died during the treatment period, however the cause of death could not be identified, as no other deaths occurred in other treatment groups or in the other generations, these were not considered to be treatment related. The F1 generation did not show any behavioral or developmental changes compared to control groups. At the two highest doses, females showed a dose-related decreased gain in bodyweight in the third week of gestation, body weight was approximately 5% less than control animals and weight-gain approximately 13% less than control; however these were not statistically analyzed. At the highest dose, there was a statistically significant decrease in mating index which the authors attributed to an atypical estrous cycle in four females from this group. The authors reported that there was a slightly lower post-implantation survival index, and that slightly fewer pups were born in females dosed with the highest concentration of lutein; however this was not statistically analyzed. Necropsy of the F1 generation did not highlight any toxicologically relevant findings. In the F2 generation there were no alterations to bodyweight gain, necropsy or histopathological findings. The authors identified a No Observed Adverse Effect Level (NOAEL) of 150 mg/kg.bw/day based on a reduced post-implantation survival in the parental and F1 generation, lower bodyweight gain in the F1 generation and reduced fertility in the F1 generation at the highest dose (EFSA, 2010a).

Special Studies

Immunology

In a 12 week immunology study in female cats, cats (56) were administered a lutein extract from marigold petals (containing approximately 77% lutein and approximately 5% zeaxanthin) at doses of 0, 1, 5 or 10 mg/day (approximately 0, 0.7, 3.5, or 7.1 mg/kg.bw/day of lutein and 0.05, 0.25, or 0.5 mg/kg.bw/day of zeaxanthin, respectively). Only bodyweight and immunological parameters were measured. The authors observed a dose-related increased in delayed-type

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hypersensitivity response to vaccine. At the end of the study, in animals administered the highest dose, there were significantly increased percentages of CD4+ and CD21+ cells. In these animals, plus the animals from the mid-dose, concentrations of IgG were also significantly higher from week 8. There were no differences in the concentration of IL-2 in control or treated animals. The authors concluded that dietary lutein stimulated cellular and humoral immune response in cats; a NOEL was not specifically identified (Kim et al., 2000a). In 2009, this study was used by the NRC as the basis for the risk assessment of lutein in cats.

In a study conducted by the same authors and utilizing a similar study design, beagles (14/female/dose) were fed diets containing a lutein extract from marigold petals (containing approximately 77% lutein and approximately 5% zeaxanthin) at doses of 0, 1, 5 or 10 mg/day (approximately 0, 0.4, 0.9, 1.75 mg/kg.bw/day for lutein and 0.03, 0.06 and 0.12 mg/kg.bw/day for zeaxanthin, respectively). Only bodyweight, immunological parameters and hematology were measured. Similar to cats, there was an increase in delayed type hypersensitivity response, an increase in IgG concentration and no change in IL-2 in treated dogs. Hematology also revealed a significantly increased mitogen-stimulated proliferation of peripheral blood mononuclear cells in treated animals. In a similar conclusion to the study in cats, the authors reported that dietary lutein stimulated cellular and humoral immune response in dogs (Kim et al., 2000b). In 2009, this study was used by the NRC as the basis for the risk assessment of lutein in dogs.

Ocular Toxicity

The paucity of studies of the effects of lutein and zeaxanthin in dogs or cats, and the fact that these species lack a true macula (the major lutein-containing region of the retina) limit the number of comments that can be made regarding ocular toxicity of these compounds in dogs and cats. However, studies designed to cause retinal accumulation of lutein and/or zeaxanthin have been conducted in numerous species (including humans) in which retinal accumulation of these compounds does occur (Leung et al., 2004; Dilsiz et al., 2006; Chew et al., 2014). None of these studies has defined a dose at which ocular toxicity occurs. This is true even with chronic ingestion. In one such study, female rhesus monkeys were fed either 9.34 mg lutein/kg and 0.66 mg zeaxanthin/kg, 10 mg zeaxanthin/kg, or 0.5 mg/kg each of lutein and zeaxanthin once daily for 12 months and were assessed 12, 18, and 24 months after supplementation was started. Although mean plasma and ocular tissue concentrations of these carotenoids and their metabolites were elevated in supplemented monkeys, no evidence of ocular or renal toxicity was noted (Khachik et al., 2006). Even a trial in which 0.75 mg (250 µL x 0.3% solution) of crystalline lutein and zeaxanthin was injected into the vitreous body immediately adjacent to the retina of rabbits failed to show toxicity (Casaroli-Marano et al., 2015). In this latter study, rabbits were examined clinically once daily for 7 days post injection (dpi), and electrophysiologically, histopathologically, and ultrastructurally at 7 dpi. No adverse anatomic or physiologic effects were noted. Furthermore, *in vitro* cytotoxicity assays using up to 2% lutein/zeaxanthin (along with other compounds known to be cytotoxic) showed no toxic effect on any ocular cell lines studied over different test times ($IC_{50} > 0.135\%$).

Kalariya, *et al.* assessed effects of synthetically-created lutein breakdown products (LBP) - not lutein itself - on a cultured line of human retinal pigment epithelial (RPE) cells (Kalariya et al., 2009). Although their data suggest that breakdown products of dietary carotenoids could be genotoxic in this line of cultured RPE cells and that LBP-induced genotoxic effects could worsen

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oxidative stress, it is unclear how these *in vitro* conditions would be moderated by robust and protective homeostatic and repair mechanisms expected in *in vivo* situations. Likewise, the authors acknowledge that the concentrations of carotenoid breakdown products (CBP) they tested were higher than those achieved in humans with short term xanthophyll supplementation. Additionally, it is unclear whether the specific CBPs created by *in vivo* oxidation in this study are similar in diversity and interactions to those that may occur physiologically or with lutein supplementation. Finally, due to the lack of *in vivo* work in non-primate species, it is unclear whether the situation in dogs or cats would mimic that in humans, especially given the lack of a true macula in these species. These questions are particularly important given that the authors showed that the genotoxic effects were dose-dependent and time-dependent.

Taken together, these data predict that it is unlikely that lutein and/or zeaxanthin fed at 1.8 mg/kg.bw/day would achieve levels expected to cause ocular toxicity.

Cytotoxicity
The use of oxidized lutein in a cell culture model examined the occurrence of antioxidant and cytotoxic activities. (Lakshminarayana et al., 2013). The work indicated that normal cells were unlikely to be affected, based on the concentrations of BHT, oxidized lutein, and lutein under the conditions of study. Also noted was that antioxidant and cytotoxic properties of the compounds may be beneficial and support anticancer properties (such as cell proliferation).

Evaluations by Authoritative Bodies

JECFA
In 2006, the Joint FAO/WHO Executive Committee on Food Additives (JECFA) evaluated lutein as prepared from *Tagetes* preparations with a high lutein content (>80%). JECFA established an Acceptable Daily Intake (ADI) of 0-2 mg/kg.bw/day based on a NOAEL of 200 mg/kg.bw/day and using a safety factor of 100. The NOAEL was identified from a 13-week study in rodents of which this was the highest dose administered. JECFA acknowledged that the ADI was based on a short-term study; however, given the lack of effects at higher doses no additional safety factor was felt necessary (JECFA, 2006).

In the same year as the JECFA lutein evaluation, JECFA also evaluated the isomeric isomer of lutein, zeaxanthin, and concluded that due to the structural and physiological similarities and the toxicological data, a group ADI was recommended.

NRC
In 2009, the NRC reviewed lutein as a dietary supplement in pet food as per a request from the Center for Veterinary Medicine (CVM) of the FDA (National Research Council, 2009). The NRC review provides the basis for the safety assessment of lutein in this GRAS submission and only summary information taken from the review is provided below. A literature search for new studies in the target species published since the NRC report in 2009 was conducted. However no further studies were identified. The 2009 NRC review identified a Historical Safe Intake (HSI) and a Presumed Safe Intake (PSI) in cats and dogs (Table 18).

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Table 18: Historical Safe Intake (HSI) and Presumed Safe intake (PSI) of lutein in dogs and cats (Table adapted from NRC, 2009)		
Species	**HSI (mg/kg.bw/day)**	**PSI (mg/kg.bw/day)**
Dogs	**0.45**	**1.80**
Cats	**0.85**	**7.20**

The NRC defined the HSI to be the estimated intake of lutein in the target species from cat and dog food that was naturally present due to its occurrence in a number of commodities such as corn and corn gluten. The NRC reported the intake in dogs and cats to range up to 0.45 and 0.85 mg/kg.bw/day, respectively.

The PSI for dogs was based on a 12-week study investigating the effects of lutein on immune function in dogs. In the study, beagles (14/females/dose) were fed diets supplemented with lutein at either 5, 10 or 20 mg diet (reported to be equivalent to 0.45, 0.9 or 1.9 mg/kg.bw/day, respectively) for 12 weeks. It is not clear from the NRC report why the value of 1.9 was reduced to 1.8 mg/kg.bw/day.

The PSI in cats was based on a 12 week immunology study in cats (14/females/dose) fed diets supplemented with lutein at doses of 1, 5, or 10 mg/kg diet (reported to be equivalent to 0.71, 3.6 or 7.2 mg/kg.bw/day, respectively).

European Food Safety Authority (EFSA)
Lutein was re-evaluated as a food additive by the European Food Safety Authority (EFSA) in 2010. EFSA derived an ADI of 1 mg/kg.bw/day using a NOAEL of 200 mg/kg.bw/day (the highest dose in the study) from a 90-day study in rodents and applying a safety factor of 200. The study used was the same pivotal study as identified by JECFA, however EFSA used an additional safety factor as no multi-generation or chronic/carcinogenicity studies were available (EFSA, 2010b).

In 2008, the EFSA evaluated the safety, bioavailability, and suitability of lutein in infant and follow-on formulae. At the levels proposed for inclusion (250 and 500 mg/l in infant and follow-on formulae, respectively), EFSA concluded that there was no safety concern. The estimated actual intake based on this inclusion in mg/kg.bw/day was not reported (EFSA, 2008b).

EFSA have also evaluated a number of health claims related to lutein supplementation in the diet. These are not reported here as they do specifically address the safety of lutein.

In 2009 lutein was evaluated by EFSA for its use as a coloring agent for poultry at up to 80 mg/kg complete feed. EFSA concluded that "... given the widespread natural occurrence of the compound, and considering the molecular structure of the carotenoid, the FEEDAP Panel does not see reasons for concern." (EFSA, 2009).

In 2012, zeaxanthin was evaluated by EFSA on its safety as an ingredient in food supplements. EFSA used a NOAEL of 150 mg/kg.bw/day identified from a two-generation reproduction study

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in rats, and applied an uncertainty factor of 200 (100 for inter- and intra-species variation and 2 for incomplete dataset) to derive an ADI of 0.75 mg/kg.bw/day (EFSA, 2012c).

In 2009, zeaxanthin was evaluated by EFSA for its use as a coloring agent for poultry with levels estimated to be up 12 mg zeaxanthin/kg. EFSA concluded that "... given the widespread natural occurrence of the compound, and considering the molecular structure of the carotenoid, the FEEDAP Panel does not see reasons for concern." (EFSA, 2009).

Summary Risk Assessment
Lutein and zeaxanthin are isomers of each other and reported to have a similar toxicological profile, therefore a group PSI of these chemicals is recommended. Lutein is reported to have a low acute oral toxicity in rodents with no evidence of skin or eye irritation in rabbits. Acute and sub-acute studies with lutein in multiple laboratory species have not highlighted any toxicological concern at the doses administered. There was no evidence of genotoxicity; however no carcinogenicity studies were available. There was no evidence of developmental toxicity from lutein; however, in a 2-generational study with zeaxanthin there was some evidence of reduced fertility at high doses (500 mg/kg.bw/day). In line with the NRC evaluation of lutein, a group PSI for lutein and zeaxanthin of 1.8 and 7.20 mg/kg.bw/day is recommended for dogs and cats, respectively.

Based on a 1.5 kg puppy requiring 378 kcal/day, the intake of lutein (b) (4) was calculated to be 1.26 mg/kg.bw/day, total intake including that from zeaxanthin was 1.8 mg/kg.bw/day, this is the calculated PSI. Based on this, adverse effects are not anticipated and lutein and zeaxanthin are not expected to cause adverse effects.

VI.B.2 C_{21}-C_{31} Alkanes

There are no standard toxicity tests that address solely the n-alkane plant waxes present in marigold. However there are extensive reports of consumption of n-alkanes from other materials, none of which have shown any negative responses; these should be considered when evaluating the toxicity of n-alkanes from marigold.

Lutein extracts
Lutein extracted from *Tagetes erecta* may have remaining plant waxes present. The JECFA specification for lutein describes a minimum total carotenoid concentration of not less than 80%. The purity of the lutein extract also reports the presence of not more than 14% plant waxes; these are later described in the "Tests" section as n-alkanes C25-35 (JECFA, 2004).

In assays addressing the toxicity or pharma-coactive properties of lutein, lutein extracts from *Tagetes erecta* have been used (Chew et al., 1996; Gradelet et al., 1996; Kim et al., 2000b, a). From the concentration of lutein present in these extracts, it is apparent that other botanical ingredients from lutein were also present. Although these studies were not standard toxicity tests, no adverse effects were reported.

Other plant materials
Plant waxes including those identified in II.C.3 are endogenous to nearly all terrestrial plants. The exact n-alkane profile of the plant waxes may be dependent upon a range of factors

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including the species, growing region, and season. Intake of these waxes may occur from a variety of different plant materials such as legumes, grains, nuts, and vegetables (JECFA, 2003). In humans, intake of these alkanes from natural components of food has been estimated to be 0.5 mg/kg.bw/day (97.5th percentile: 0.8 mg/kg.bw/day)(JECFA, 2003).

Other waxes also commonly used in the food industry may contain n-alkanes, the JECFA specification for carnuba wax also defines the presence of a number of straight chain hydrocarbons (numbered from C27-C31) (JECFA, 1998).

Mineral hydrocarbons

n-alkanes may also be found as a component of mineral oils alongside naphthenes (alkyl-substituted cyclo-alkanes, and aromatics (polyaromatic hydrocarbons). Although, by definition, these straight-chain hydrocarbons are derived from non-vegetative sources, the chemical identity of the n-alkanes is the same (although their profile may be different). Mineral oils have been evaluated by a number of authoritative bodies including JECFA and EFSA. These were chemically defined as being refined products (free of unsaturated, or aromatic structures), with a minimum carbon number of 25 or C10-C25, respectively. A group ADI for high viscosity paraffins of 20 mg/kg.bw/day was recommended based on a NOEL of 2000 mg/kg.bw/day from a 90 day study in rodents. A lower ADI of 10 mg/kg.bw/day was recommended for medium viscosity based on a further 90-day study in rodents which identified a NOEL of 1200 mg/kg.bw/day with an applied safety factor of 100 (JECFA, 2003).

The EFSA evaluation of mineral oils in 2012 addressed a number of concerns that had been raised regarding the mutagenicity of mineral oils. EFSA concluded that these were related to the presence of PAHs and not from the straight chain n-alkanes that were also present in plants. EFSA stated that the rate of absorption from the gut of linear alkanes (n-alkanes) reduced as the carbon number increased with n-alkanes of carbon numbers > C29 not significantly absorbed and only low absorption of the n-alkanes typically found in plant waxes. EFSA did not identify an ADI for mineral oils due to the complexity of the mineral oil mixtures and absence of toxicological studies on the mixtures to which humans are typically exposed. Instead, they advised a MOE approach using a NOAEL of 19 mg/kg.bw/day based on a rodent study conducted with medium-viscosity hydrocarbon that identified hepatic micro granulomas (EFSA, 2012b). This is considered over-conservative in light of the straight-chain, longer length n-alkane found in plant waxes.

Refined mineral oils are also used as adjuvant in vaccines, bases, ointments, and drug vehicles in pharmaceuticals and veterinary medicines. Calculated doses have been estimated to be up to approximately 1,500 mg/kg.bw/day without evidence of adverse effects in humans. No estimated intakes have been calculated for livestock or pets (EMEA, 1995).

Summary Risk Assessment

Given that intake of n-alkanes may occur from a number of plant-based materials, the limited absorption from the gut and studies which administered lutein extracts without adverse effects, n-alkanes are considered to be of low food safety risk. The intake of n-alkanes in a 1.5 kg puppy has been calculated to be 1.5 mg/kg.bw/day, which, under these circumstances, is considered GRAS.

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VI.B.3 Free Fatty Acids

Linoleic, palmitic, oleic, myristic, and stearic acid have been identified in marigold extract (Sujith et al., 2010; Gong et al., 2011). These five fatty acids are common components of vegetable and animals fats. They have been extensively reviewed in the public literature and by authoritative bodies such as JECFA. High levels are reported in common components of human food and animal feed such as vegetable oils (81.5 g/100 g), beef byproducts (11.8 g/ 100 g) or rice bran (4.17 g/100g) (USDA National Nutrient Database for Standard Reference, 2015). Adverse effects have not been reported when consumed as part of a nutritionally complete and balanced diet. Royal Canin's pet food is compliant with NRC energy requirements (National Research Council, 2006). As such, the total amount of fatty acids from marigold extract as well as from other components in the finished product is considered safe. Linoleic acid is also recognized as an essential requirement in mammals, the NRC have set a Safe Upper Limit (SUL) of 16.4 g and 13.8 g per 1000 kcal for adult dogs and cats, respectively (National Research Council, 2006). The maximum concentrations of linoleic acid determined in the marigold extract and in finished product are compliant with NRC guidelines. The concentration of free fatty acids for a 1.5 kg puppy is 6.36 mg/kg.bw/day. At these concentrations no adverse effects are expected and therefore are considered GRAS.

VI.B.4 Gums & Resinous Matter from Dried Marigold

Gums are typically metabolized into their sugar constituents (arabinose, galactose, glucose, mannose, etc.), fermented in the gut into low-molecular weight fatty acids (acetic, propionic, butyric), and excreted unchanged, or some combinations of these. The gum extracted from marigold seed petals contains arabinose, galactose and glucose in the ratio 15:3:7 (Medina and BeMiller, 1993). There is no safety concern with these sugars. Many gums are not metabolized by mammals; they are excreted unchanged and do not present a safety issue. JECFA has evaluated a number of gums through the years and for most of them, has allocated an "ADI not specified," meaning that the gums are considered to be amply safe at their indicated use levels without the need for issuance of a safety tolerance (JECFA). From the information available on the gums in the extract, it is expect that the same would be true of the gums in marigold extract and that the low levels anticipated from the proposed use here would be GRAS.

The resins in the marigold extract are based on terpenes and terpene ketones and comprise a significant part of the essential oils which produce the aroma of marigold flowers. A series of monoterpenes and terpenoids were screened for toxicity by Environmental Protection Agency (EPA) as a part of its "High Production Volume (HPV) Challenge Program." (US EPA, 2009). The repeat-dose toxicity of virtually all of these resins was approximately 1000 mg/kg.bw/day, indicating human ADIs of approximately 10 mg/kg.bw/day or 600 mg/person/day. Lower molecular weight terpenes and their oxidation products, terpenoids, are the essential oils of many types of plants and flowers. The chemicals in the essential oils, occur naturally at 10-20 milligram levels in servings of many common foods and are the principal ingredients in many flavorings. For example, the mono-cyclic terpene characteristic of citrus is d-limonene; the principal active agent in the food ingredient "orange zest". Limonene is 3-14% by weight of the orange peel, therefore 2 g of orange zest (a typical use level in an orange-flavored cake), contain approximately 200 mg of limonene. Accordingly, the low levels of dietary exposure to terpenes from the intended use of marigold extract are considered to be GRAS.

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VI.B.5 Silicon dioxide

As stated in section IIA, silicon dioxide is not intrinsic to marigold extract, but has wide spread use across the feed industry as an anticaking agent. It has been evaluated by a number of authoritative bodies including JECFA and EFSA where is it considered to be a low food safety concern, and has GRAS in the USA for a variety of uses. Silicon dioxide may be safely used in animal feed as an anticaking agent under 21 CFR 573.940. AAFCO Feed Ingredient Definition #87.3 also provides for its use up to 50% in vitamin E supplements. The intended use of silicon dioxide in Cuatroxan is (b) (4) That appears well within the specification of 21 CFR 573.940(c), which limits the inclusion of silicon dioxide to not more than 2% of the finished food.

Due to the low food safety risk from silicon dioxide it has not been considered further for toxicological risk assessment. As a component of the silicon dioxide, sodium sulfate is expected to break into its respective ions, both of which are commonly found in feeds. Therefore, it is not necessary to consider this further in terms of toxicological risk assessment.

VI.B.6 Vegetable Oil

Vegetable oil has widespread use in the food and feed industry, it is not necessary to consider this further in terms of toxicological risk assessment

VI.B.7 Water

It is not necessary to consider water in terms of toxicological risk assessment.

VI.B.8 Rosemary extract

Rosemary (*Rosmarinus Officinalis*) extract may contain multiple chemical constituents, that may vary dependent upon the extraction process as well as the part, growing conditions and origins of the plant (Viuda-Martos et al., 2007; Genena et al., 2008). The rosemary extract added to the marigold extract described in section II.C.9 is extracted by organic solvents including acetone and methanol. The extraction method produces an extract that typically contains 30% carnosol/carnosic acid (US Patent, 2002) which is considered the active ingredient in rosemary. For this risk assessment, toxicity data for rosemary or rosemary extract rather than individual materials are used. Where the authors have reported composition of the extract or extraction procedure, this is also reported to allow comparison between rosemary extracts used.

Acute Toxicity

Rosemary extract (ethanol extraction, 7 -10% carnosol/carnosic acid) is of low acute oral toxicity with LD_{50}s greater than 8.5 or 10 g/kg.bw in male or female mice respectively (EFSA, 2008a).

Irritation and Sensitization

Rosemary leaf oil applied at full strength was moderately irritating to rabbit skin after application for 24 hours to intact and abraded skin under occlusion (Opdyke, 1974).

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Metabolism and Pharmacokinetics
No studies were located.

Repeat Dose Toxicity
In a 5-day study, mice (number not reported), were administered rosemary extract (ethanol extraction, 7-10% carnosol/carnosic acid) via gavage at doses of 4.3 or 5 mg/kg.bw/day in male or females, respectively. After 5 days, animals were sacrificed and necropsied. The authors did not observe any changes in behavior or food or water intake. Body weight increased in males but did not change for females. Males also showed a higher incidence of hepatic lipidosis (no further details reported) (EFSA, 2008a).

In a 90-day study in Sprague Dawley rats (number of animals not reported), rats were fed rosemary extract (ethanol extraction, 7-10% carnosol/carnosic acid or ethanol extraction followed by deodorization). Diets were designed to provide doses of 0, 500, 1500 or 5000 mg/kg diet (reported to be 0, 40, 120 or 400 mg/kg.bw/day, respectively). In animals fed the highest dose of ethanol-extracted rosemary, there was a decrease in food uptake which resulted in a slight reduction in body weight gain. The authors attributed this to a palatability problems as food uptake, and consequently reduction in weight gain, was not observed in animals administered deodorized rosemary extract. All concentrations and extracts of rosemary assessed were associated with increased liver weight, and this followed a dose-response relationship. However, no changes in absolute liver weight were seen. Hepatic histopathology did not reveal gross or microscopic changes, however a statistically insignificant increase in liver enzymes (P450) was observed. The authors reported a NOAEL of 5000 mg/kg diet (400 mg/kg.bw/day) because the observed hepatic changes were not considered adverse but, rather, due to an adaptive response (reported in EFSA, 2008a).

In a follow-up study, designed to replicate the effects seen in the previous study and to show that changes were reversible/adaptive, female rats were fed rosemary extracts (supercritical CO_2 extraction) for 91 days at a dose of 2400 mg/kg diet (reported as 195 mg/kg.bw/day) followed by a recovery period of 28 days before study termination. Liver weight was slightly increased compared to the control group and to results of previous study; Histopathology revealed minimal centrilobular hypertrophy. Increased activity of P450 hepatic enzymes, in particular CYP2A, CYP2C11, CYP2E1 and CYP4A was also reported. After the recovery period, liver weight and enzyme activity returned to values similar to those seen in control animals. The authors concluded that the increased liver weight accompanied by centrilobular hypertrophy and enzyme induction was a reversible adaptive. Consequently, the authors identified a NOAEL of 2400 mg/kg diet (reported to be 195 mg/kg.bw/day) for rosemary extract from supercritical CO_2 extraction (reported in EFSA, 2008a).

In a 13-week study, rats (20/gender/dose) were given one of two rosemary extracts: rosemary extract from acetone (approximately 10% carnosic acid) or rosemary extract from supercritical CO2 extraction (approximately 30% carnosic acid). The supercritical CO_2 rosemary extract was provided in the diet so as to provide doses of 300, 600, or 2400 mg/kg diet, or approximately 15, 30 or 180 mg/kg.bw/day, respectively. The authors reported this as 180 mg/kg.bw/day. The acetone rosemary extract was provided at 3800 mg/kg diet (approximately 190 mg/kg.bw/day). Some alterations to clinical chemistry were observed, however these were inconsistent and not

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dose-related and therefore the authors considered these to be normal variation and not treatment-related. At the highest dose of rosemary extract (supercritical CO_2 extraction), animals displayed decreased body weight and food consumption. Because no other adverse effect was observed, the authors attributed this to palatability issues rather than a toxicity-related change. The authors identified a NOAEL of 2400 mg/kg diet (reported as 180 mg/kg.bw/day) for the acetone rosemary extract and a NOAEL of 3800 mg/kg diet (reported as 190 mg/kg.bw/day) for the supercritical CO_2 rosemary extract (reported in EFSA, 2008a).

Genotoxicity
Overall, there is limited evidence of genotoxicity.

Table 19: *In vitro* Assays

Rosemary extract	Assay	Metabolic activation	Result	Reference
Supercritical CO_2	**Reverse mutation assay in *Salmonella typhimurium* (strains TA98, TA100, TA102, TA1535 and TA1537)**	+/-	**Negative**	**EFSA, 2008**
Ethanol extract	**Reverse mutation assay in *S. typhimurium* (strains TA98, TA100, TA 1535, TA1537, TA1538)**	+/-	**Negative**	**EFSA, 2008**
Ethanol extract	**Chromosomal aberration assay in human lymphocytes**	+/-	**Negative**	**EFSA, 2008**
Not defined	**Reverse mutation assay in *S. typhimurium* (strain TA98)**	+/-	**Negative**	**Zegura *et al.*, 2011**
Not defined	**Comet Assay (HepG2)**		**Negative**	**Zegura *et al.*, 2011**

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Table 20: *In Vivo* Assays			
Rosemary extract	**Assay**	**Result**	**Reference**
Rosemary oil	**Comet assay (Swiss mice)**	**Positive**	**Maistro *et al.*, 2010**
Rosemary oil	**Micronucleus assay (Wistar rats)**	**Positive**	**Maistro *et al.*, 2010**
Water: alcohol	**Micronucleus assay (Wistar rats)**	**Negative**	**Gaiani *et al.*, 2006**
Absolute ethanol	**Micronucleus assay (Swiss albino mice)**	**Negative**	**Fahim et al., 1999**
Rosemary oil	**Micronucleus assay (Swiss mice)**	**Negative**	**Furtado *et al.*, 2008**
Rosemary oil	**Chromosomal aberration assay (Wistar rats)**	**Positive**	**Maistro *et al.*, 2010**
Water: alcohol	**Chromosomal aberration assay (Wistar rats)**	**Negative**	**Gaiani *et al.*, 2006**
Hexane: ethanol extract	**Micronucleus assay (OF1 mice)**	**Negative**	**EFSA, 2008**

Carcinogenicity
No studies were located.

Reproductive and Developmental Toxicity
In a developmental study, Wistar rats (12 -14 females/dose) were fed via gavage 26 mg of 30% aqueous extract of rosemary extract (reported to be equivalent to 39 mg/kg.bw/day of rosemary). Animals were fed for Days 1 to 6 or 6 to 15 of gestation and sacrificed on Day 21 of gestation. There was no increased incidence of abnormalities or delayed development in treated fetuses compared to controls. The authors reported a higher incidence of post-implantation losses in the dams treated from Days 1 to 6 of gestation; however, this was not statistically significant. The authors did not report a NOAEL but concluded that rosemary extracts may have an anti-implantation effect. However, as the effect was not statistically significant and therefore may be due to natural variation, it is not considered appropriate to attribute this affect to rosemary, and a NOEL of 39 mg/kg.bw/day can be identified based on the highest dose in the study (Lemonica et al., 1996).

In a fertility study, male Wistar rats (30 males/dose) were administered *Rosmarinus Officinalis* extract (no further details reported) via gavage at doses of 50 or 100 mg/kg.bw/day for 60 days. Spermatological parameters (including sperm dynamics, and histometric parameters of the sperm), serum testosterone levels and testicular weight were analyzed at the end of the treatment period. Animals from both treatment groups displayed statistically significantly decreased serum testosterone levels and increased spermatocytes. At the highest dose there was also an increase in

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the number of leydig cells and spermatids. The authors concluded that *Rosmarinus officialis* may have anti-androgenic effects but did not identify a NOAEL (Heidari-Vala et al., 2013). Based on the report, although a decrease in testosterone levels was observed, there were no changes in total sperm count, or mobility, or viability and therefore the biological significance of these results is not clear.

In a fertility study, male mice were fed rosemary extract (ethanol: water) for 63 days in their drinking water at doses of 250 or 500 mg/kg.bw/day. On Day 53, each male mouse was housed with two untreated females for 10 days and then sacrificed on Day 63; females were sacrificed 1 week after removal of the male rats. There were no changes in body weight or behavior at any doses. At the highest dose there was a significant decrease in the average weight of the epididymides, ventral prostate glands, seminal vesicles or vas deferens. There was also a significant decline in spermatogenesis, with associated decline in sperm motility and density. Also, animals administered the highest dose had decreased testosterone levels. In females mated with males from the highest dose group there was a significant decrease in the number of pregnant females, the number of implantations and viable fetuses, and a significant increase in the number or resorptions. A similar trend was reported in females mated with the males from the low dose group; however it was not statistically significant (Nusier et al., 2007). Based on the reported findings, a NOAEL of 250 mg/kg.bw/day can be identified for male rats based on effects upon reproductive function.

Other studies

Various studies have reported potential health benefits of rosemary (including rosemary extracts and oil) administered at doses up to 500 mg for 16 weeks in rats (Ibarra et al., 2011); however these studies do not follow toxicological study designs and do not specifically report standard toxicological endpoints such as histopathology or hematology and therefore are not suitable for use in the risk assessment.

Evaluations by Authoritative Bodies

In 2008 EFSA evaluated extracts of rosemary prepared by different extraction techniques and proposed for use as an antioxidant in food. The EFSA concluded that there was insufficient toxicological data to establish an ADI, however the existing data did not give reason for concern. (There were no reproductive and developmental long term studies). Based on rosemary intake in the European population and using pre-school children as the worst case scenario, carnosol intake for the 97.5th percentile was estimated to be 0.23 mg/kg.bw/day. The EFSA recognized that the safety margin between estimated intake and the identified NOAELs for rosemary extract (180-400 mg/kg.bw/day) and carnosol (20 - 60 mg/kg.bw/day) were at least 88 (EFSA, 2008a).

As per European Union (EU) regulation 1831/2003, EFSA evaluated an application for a rosemary extract (defined as 9-10% carnosic acid) to be added as a technological additive (antioxidant) for cat and dog feed. However EFSA did not deliver a final opinion on the extract as the applicant did not provide additional information requested by EFSA and consequently, the extract was not approved for use (EFSA, 2012a).

Rosemary is listed by the Association of American Feed Control Officials (AAFCO) under section 582.10 Spices and other Natural Seasonings and Flavorings, and section 582.20 Essential

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oils, Oleroresins (solvent-free) and Natural Extractives (Including distillates) as a Source of Flavor (AAFCO, 2013).

Summary Risk Assessment

Rosmarinus Officialis is a commonly-used herb with a long history of cultivation and use in humans. Many compounds have been isolated from rosemary, and the concentration of these may vary in extracts depending upon the extraction method. No studies with rosemary or its extract were located in dogs or cats; however based on studies in rodents, rosemary extracts were of low acute oral toxicity. Sub-chronic studies identified a range of NOELs from 180 to 400 mg/kg.bw/day based on the highest doses used in these studies. Increased liver weight was observed in these studies but the effects were considered an adaptive rather than a toxic response. The 2008 EFSA evaluation of rosemary extracts considered these studies in their review and concluded that rosemary extracts did not raise a safety concern. No long-term or carcinogenicity studies were available and there are no results from *in vivo* genotoxicity assays. However, in light of the history of use in humans, it is considered unlikely that *Rosmarinus officialis* presents a high potential for carcinogenicity. Since the EFSA review a number of reproductive and developmental studies have been published all of which have indicated that at high doses, rosemary extracts may have a negative effect on fertility and reproduction with anti-androgenic effects observed in male mice and evidence of effects on implantation in pregnant females. However, it is not clear which chemical or chemicals are responsible for this effect or their mode of action. These studies were conducted in rodents only, and not to standard OECD guidelines; it is not clear whether this is a species-specific effect or an artifact of the study. Until further studies are conducted to prove otherwise, it is considered prudent and in line with the conservative approach of risk assessment to assume that this may also affect other species. Therefore, the most appropriate study is a sub-chronic study in male rats which identified a NOAEL of 250 mg/kg.bw/day based on decreased weight of reproductive organs, sperm count, and testosterone. A further study in rats identified a lower dose based on decreased testosterone; however no effects on sperm mobility or viability were seen, so the biological significance is not clear. Equally sub-chronic studies in rats identified lower NOAELs however these were based on the highest doses in the studies and were considered overly-conservative based on the history of use in man. The NOAEL of 250 mg/kg.bw/day was based on an ethanol extraction. Although the study authors did not characterize their extract, based on the reported levels in the EFSA evaluation, a carnosol/carnosic acid content of approximately 10% is anticipated. Therefore a NOAEL for carnosic acid of 25 mg/kg.bw/day can be estimated. By applying a safety factor of 100 (for inter- and intra-species variation) to the NOAEL, a PSI for carnosic acid of 0.25 mg/kg. bw/day can be derived. Although no long term studies were available, it is not considered appropriate to apply a further safety factor to account for this as it would be considered over conservative given the history of safe use in humans.

Based on a 1.5 kg puppy consuming 308 kcal/day, the intake of carnosic acid was calculated to be 0.17 mg/kg.bw/day. Carnosol concentrations were not analyzed explicitly but instead were grouped under phenolic diterpenes where an intake of 0.03 mg/kg.bw/day was anticipated. Although other phenolic diterpenes are likely to be present, assuming a worst-case scenario and only carnosol being present, the combined intake is below the PSI and adverse effects are not anticipated. This ingredient is therefore considered GRAS.

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VI.C Summary of Toxicological Risk Assessment for the Substance

There are insufficient studies to evaluate the safety of marigold extract as a whole component, therefore its toxicity has been considered in light of the natural constituents within it. The xanophylls present were predominantly lutein, with zeaxanthin as a minor component. Due to their chemical similarity and because they are likely to have a similar toxicological profile and mode of action, lutein and zeaxanthin were grouped together and the PSI from the NRC adopted. The calculated intake of lutein and zeaxanthin was below the PSI and therefore adverse effects are not anticipated. The other major components within marigold extract were plant waxes and fatty acids; these are commonly found in many feed ingredients where they have not presented adverse effects to consumers. A large fraction of the marigold extract was composed of gums and resins. Similar to plant waxes and fatty acids, many of the anticipated gums found in marigold extract are likely present in other feed materials. They are considered to be of low food risk and in the levels present in the marigold extract are not considered to cause adverse effect. Resins including essential oils are also present in the marigold extract. Like other components of lutein; many of the oils are also present in other botanical ingredients that are commonly found in feed. Although the exact composition and concentration of these were not determined, the oils are likely to be present at very low levels and are not anticipated to cause adverse effects. Additionally, many lutein extracts have components of marigold remaining in them, where these have been used in experimental studies, adverse effects have not been reported. Based on these evaluations and the intended use level and method of marigold preparation, adverse effects are not anticipated for marigold extract. Marigold extract is available as Cuatroxan marketed by Kemin; this formulation also contains a rosemary extract. Although this GRAS submission is for marigold extract, and not all extracts will contain rosemary extract, the intake levels of rosemary extract have also been considered in the risk assessment. Similar to marigold, many natural botanical constituents are present in the extract. Based on the compositional analysis, the calculated intakes were below PSIs and adverse effects are not anticipated.

VII. OVERALL SUMMARY

In summary, there are no indications from the published literature that any adverse effects would result from the consumption by dogs and cats of marigold extract at the proposed levels. Overall, when viewed in its entirety, the scientific data summarized herein support the safety of marigold extract under the intended conditions of use as a nutritional source of lutein for dogs and cats. In accordance with the Federal Food, Drug, and Cosmetic Act ("FD&C Act") and its implementing regulations as administered by the FDA, we have determined that Royal Canin's intended use of marigold extract (*i.e.*, extracts of *Tagetes (T.) erecta*), when used for nutritional purposes by Royal Canin in diets and treats for dogs and cats at a level sufficient to provide no more than 5 mg lutein per 1000 kcal metabolizable energy is safe, and that there is a reasonable basis to conclude that this intended use is generally recognized as safe (GRAS). Thus, it may be properly concluded that marigold extract is exempt from the premarket approval requirements of the FD&C Act, and as a GRAS substance, marigold extract may be used as intended in dog or cat food at the proposed use levels in compliance with the FD&C Act and all applicable regulations.

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VIII. CONCLUSION

We, the Expert Panel, have independently and collectively critically evaluated the data and information summarized above and conclude that marigold extract is Generally Recognized as Safe (GRAS), based on scientific procedures, under the conditions of intended use as a nutritional source of lutein in dog and cat diets and treats at levels sufficient to provide no more than 5 mg per 1000 kcal metabolizable energy, as described herein.

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GRAS EXEMPTION CLAIM FOR MARIGOLD EXTRACT DERIVED FROM Tagetes erecta

David A. Dzanis, DVM, PhD, DACVN
Regulatory Discretion, Inc.

JUNE 3, 2016
Date

David J. Maggs, BVSc, DACVO
University of California-Davis

Date

Helen Clegg, PhD
Mars Petcare, Inc.

Date

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David J. Maggs, BVSc, DACVO
University of California-Davis

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Helen Clegg, PhD
Mars Petcare, Inc.

6/6/2016
Date

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GRAS EXEMPTION CLAIM FOR MARIGOLD EXTRACT DERIVED FROM Tagetes erecta

David A. Dzanis, DVM, PhD, DACVN,
Regulatory Discretion, Inc.

Date

David J. Maggs, BVSc, DACVO
University of California-Davis

May 31st 2016
Date

Helen Clegg, PhD
Mars Petcare, Inc.

Date

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Appendix A: *Curricula vitae* for Panel Members

DAVID A. DZANIS, DVM, PhD, DACVN

DAVID J. MAGGS, BVSc, DACVO

HELEN CLEGG, PhD

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Appendix B: Product Specification Sheets for Cuatroxan and Nutri-Gold Yellow

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Appendix C: Communications with CFSAN Regarding Requirement for a Color Additive Petition

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Appendix D: Lutein Content of Cuatroxan and Nutri-Gold Yellow

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Appendix E: Estimated Lutein (b) (4)

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Appendix F: ORO GLO 20 Product Specification Sheet

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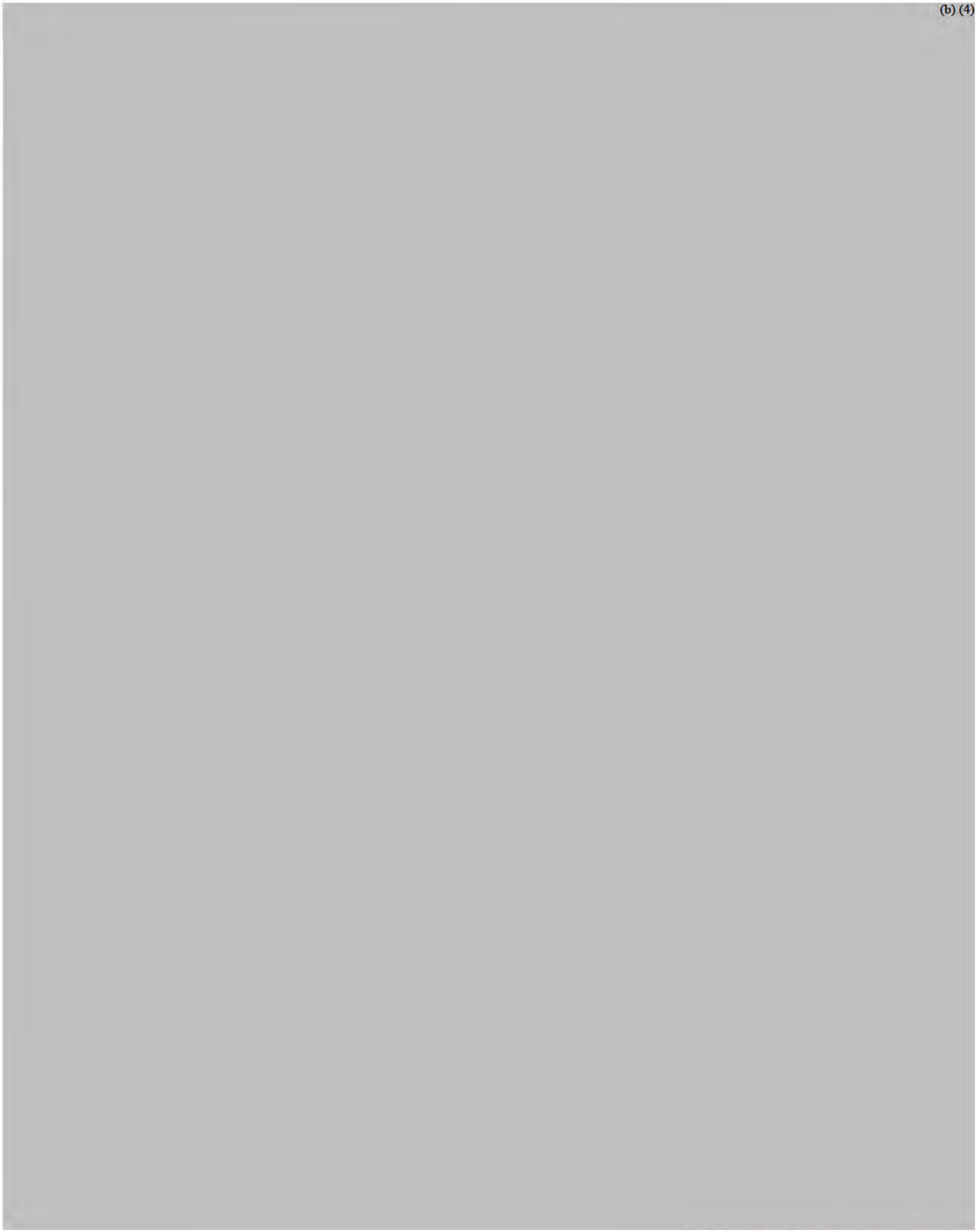
(b) (4)



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