I. COVER LETTER

August 24, 2020

Consumer Safety Officer
Office of Nutrition, Labeling and Dietary Supplements (HFS-810)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
Department of Health & Human Services
5001 Campus Drive
College Park, Maryland 20740

Dear Sir / Madam:

The undersigned, Jose Avalos, submits this NDI notification under section 413 (a)(2) of the Federal, Food, Drug, and Cosmetic Act with respect to (Astaxanthin) DSM Nutritional Product's tradename "Astasana 5% CWS/S-TG*", also referred to in sections of this document as synthetically derived "Astaxanthin crystalline" which is a new dietary ingredient.

Sincerely yours,

Jose Avalos

Jose Avalos – Primary Contact
Sr. Manager Regulatory Affairs
Jose.avalos@dsm.com
DSM Nutritional Products
6480 Dobbin Road
Columbia, MD 21045
Tel. 240-512-2617
Fax 410-740-5480

Bill Turney – Secondary Contact
Head- North America Regulatory Affairs
Bill.turney@dsm.com
DSM Nutritional Products North America

Terminology:

***Astasana™ 5% CWS/S-TG**
Astasana™ is the tradename of Astaxanthin formulated product. CWS/S-TG is DSM's acronym for "Cold Water Soluble-Tablet Grade.

Astaxanthin crystalline
Active ingredient in Astasana™† CWS/S-TG

II. TABLE OF CONTENTS

A. ADMINISTRATIVE

A.1 Detailed description of the Identity of the new dietary ingredient

Astaxanthin (Astasana™ 5% CWS/S-TG) is the carotenoid pigment that gives the flesh of salmon and trout its characteristic pink-red color. These fish derive astaxanthin strictly from the diet, through consumption of zooplankton (*e.g.*, krill), shrimp and other crustaceans in the wild, and through consumption of feed supplemented with astaxanthin at aquaculture facilities.

The chemical structure of the astaxanthin present in marine species is identical (see Figure 1) to that of astaxanthin derived through chemical synthesis. However, due to the presence of two asymmetric carbons (C-3 and C-3'), the molecule exists as three stereoisomers, two enantiomers [(3R,3'R) and (3S,3'S)]; and a meso form (3R,3'S). The distribution of the three isomers differs depending on the source, as Table 1 illustrates. In the flesh of wild-caught salmon, for example, (3S,3'S) was reported to be the predominant isomer (70-85%), followed by 12-28.2% (3R,3'R), and 2-6% of the meso form (Schiedt *et al.*, 1981; Rufer *et al.*, 2008). On the other hand, analysis of *Haematococcus pluvialis* microalgal meal revealed a distribution of 87.6% (3S,3'S), 10.1% of the meso form, and 2.3% (3R,3'R), whereas meal from the red yeast *Phaffia rhodozyma* contained 98.5% of the (3R,3'R) enantiomer and 1.5% of the meso form (Moretti *et al.*, 2006). Chemically-synthesized astaxanthin, including DSM's AstaSana™, generally consists of a mixture of 50% of the meso form and 25% each of the (3S,3'S) and (3R,3'R) enantiomers.

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Astaxanthin also has various geometrical isomers with all-*trans* astaxanthin (all-*E* astaxanthin) as the predominant one. In addition, at least two other geometrical, cis-isomers (*Z*-isomers) occur to some extent in nature and in racemic astaxanthin from chemical synthesis.

As discussed below, astaxanthin has been used as an animal feed additive for several decades. In the United States, regulation 21 CFR §73.35 permits its use in the feed of salmonid fish to enhance the color of the flesh went into effect in 1995[1].

Astaxanthin is a carotenoid pigment that has been shown to have antioxidant properties, and was the subject of a previous GRAS notice (GRN No. 294), submitted by Fuji Chemical Industry Co., Ltd. in 2009. In addition, DSM's astaxanthin was the subject of an internal expert panel determination of Safety (GRAS) for the same uses of astaxanthin identical to those described in GRN No. 294, which generated no questions from U.S. FDA.

In the European Union (EU), its use as an additive for fish is authorized under Council Directive 70/524/EEC[2]. The safety of dietary astaxanthin in humans from consumption of salmon, whether wild or farmed, has therefore been reviewed by various regulatory authorities as part of the premarket approval process.

Aside from imparting color to the flesh of fish, astaxanthin has also been shown to have antioxidant properties, similar to other carotenoids. This has led to increasing interest in supplementation, and considerable research in humans.

[1] Federal Register (1995). Listing of Color Additives Exempt from Certification; Astaxanthin. Final rule. 60 FR 18736, April 13, 1995.
[2] European Council Directive (1970). Council Directive of 23 November 1970 concerning additives in feeding-stuffs. OJ L 270, 14.12.70, p. 1.

A.2 Conditions of Use

Refer to section B3

A.3 Designation of Non-Disclosable Information

Please refer to enclosed "Redacted Copy" of this Notification. Please note that all Appendices enclosed with this notification are considered Confidential and Proprietary Information.

A.4 Safety Narrative for the dietary supplement

Astaxanthin has been used as an animal feed additive for several decades. In the United States, regulation 21 CFR §73.35 permits its use in the feed of salmonid fish to enhance the color of the flesh went into effect in 1995[1].

In the European Union (EU), its use as an additive for fish is authorized under Council Directive 70/524/EEC[2]. The safety of dietary astaxanthin in humans from consumption of salmon, whether wild or farmed, has therefore been reviewed by various regulatory authorities as part of the premarket approval process.

Aside from imparting color to the flesh of fish, astaxanthin has also been shown to have antioxidant properties, similar to other carotenoids. This has led to increasing interest in supplementation, and considerable research in humans.

In addition, In 2015, DSM obtained an Expert Panel Opinion regarding the GRAS status of our synthetic astaxanthin identical uses and use levels were considered GRAS through scientific procedure as described in the Fuji Chemical's GRAS Notice GRN 000294 for *Haematococcus pluvialis* extract containing astaxanthin esters .The permitted uses for our synthetic astaxanthin (Astasana™) as a food ingredient are in baked goods, beverages, cereals, chewing gum, coffee and tea, dairy product analogs, frozen dairy desserts and mixes, hard candy, milk products, processed fruits and fruit juices, processed vegetables and vegetable juices, and soft candy at appropriate levels depending on the food application.

[1] Federal Register (1995). Listing of Color Additives Exempt from Certification; Astaxanthin. Final rule. 60 FR 18736, April 13, 1995.
[2] European Council Directive (1970). Council Directive of 23 November 1970 concerning additives in feeding-stuffs. OJ L 270, 14.12.70, p. 1.

B. CHEMISTRY AND IDENTITY

B.1 Chemical name

The generic name of the substance under consideration is astaxanthin. Its chemical name and structure are shown in **Figure 1.**

B.2 Chemical Abstract Service Registry Number

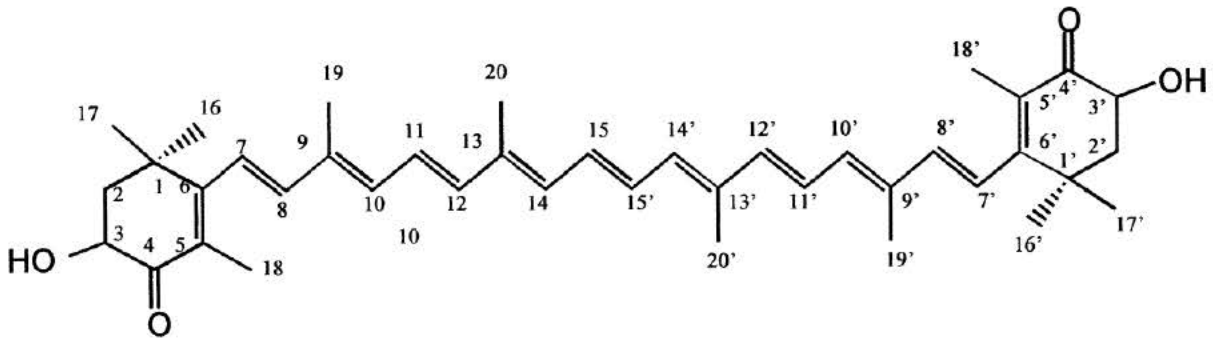
Common name: Astaxanthin

Chemical name: 3,3'-dihydroxy-β,β-carotene-4,4'-dione

CAS number(s): 7542-45-2[a]; 472-61-7

Chemical formula: $C_{40}H_{52}O_4$

Figure 1- Chemical name and structure of astaxanthin



MW: 596.86

[a] CAS Registry number of chemically-synthesized astaxanthin.

B.3 Conditions of Use

It is intended that chemically derived Astaxanthin (Astasana™) will be used as a source of astaxanthin in dietary supplements and will be sold in forms suitable for dietary supplements. The labeling for the new dietary ingredient will specify that it can be used at up to 12 mg astaxanthin/person/day to supplement dietary intake. AstaSana™ 5% CWS/S-TG is intended for use in dietary supplements in the US, It is suitable for oral dosage forms like tablets, chewables, effervescent tablets, and hard-shell capsules or other forms suitable for dietary supplement applications.

B.4 Manufacturing Process Description of the active dietary ingredient-Confidential

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(b) (4)

B.5 Active Ingredient Specifications - Astaxanthin Crystalline:

Table 2- below depicts the current Astaxanthin product specifications and results of 3 different lots. All manufactured lots met the current specifications of the active raw material Table 2 provides a summary of the results of analyses of three (3) lots of astaxanthin crystalline, which show conformance with the established specifications. Actual certificates of analysis are attached as **Appendix 1**.

Table 2 (b) (4)

(b) (4)

B.6 Additional Quality Testing Parameters- Astaxanthin crystalline

The standards for microbiological purity in astaxanthin crystalline are as follows:

Total aerobic microbial count	Max. 10^3 CFU/g
Total combined yeast/mold count	Max. 10^2 CFU/g
Enterobacteria	< 10 CFU/g
Escherichia coli	Negative in 10 g
Salmonella spp.	Negative in 25 g
Staphylococcus aureus	Negative in 10 g
Pseudomonas aeruginosa	Negative in 10 g

B.7 Residual Solvents

Table 3 (b) (4)

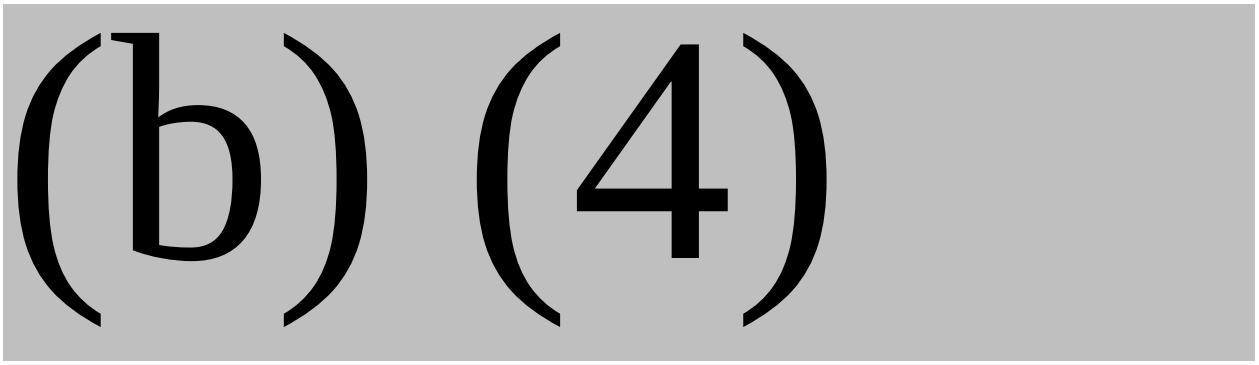
(b) (4)

C. Finished Formulated Product – Astasana™ 5% CWS/S-TG

C. 1 Description

The astaxanthin crystalline formulated product will be sold as a dietary ingredient suitable for finished dosages forms : tablets, chewables forms, effervescent tablets hard shell capsules, and other suitable dietary supplement forms under the DSM brand name Astasana™ 5% CWS/S-TG. Astasana™ 5% CWS/S-TG consists of free flowing particles (fine granular powder). They contain astaxanthin finely dispersed in a matrix of Modified Food Starch and Glucose syrup. Dl-a-tocopherol and sodium ascorbate are added as antioxidants. All additives in the formulated product are food grade.

C.2 Product Composition:



C.3 Finished Product Specifications: Astasana™ 5% CWS/S-TG

Table 4 (b) (4)

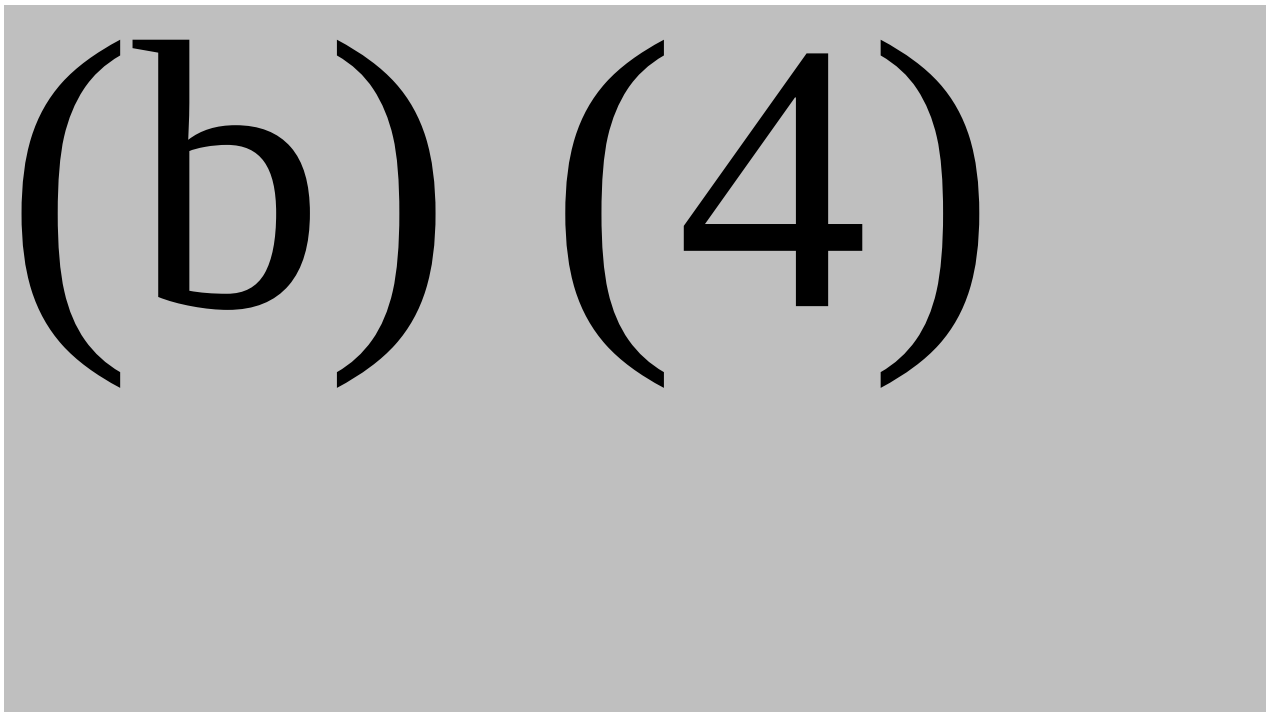


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Table 5 Microbiology Specifications

Testing	Limits	Method
Total aerobic microbial count	Max. 10^3 CFU/g	Ph. Eur.
Total combined Yeast/Moulds count	Max. 10^2 CFU/g	
Enterobacteria	< 10 CFU/g	
Escherichia coli	Negative in 10 g	
Salmonella spp.	Negative in 25 g	
Staphylococcus aureus	Negative in 10 g	
Pseudomonas aeruginosa	Negative in 10 g	

C.4.2 Residual solvents Specifications finished Product Astasana™ 5% CWS/S-TG

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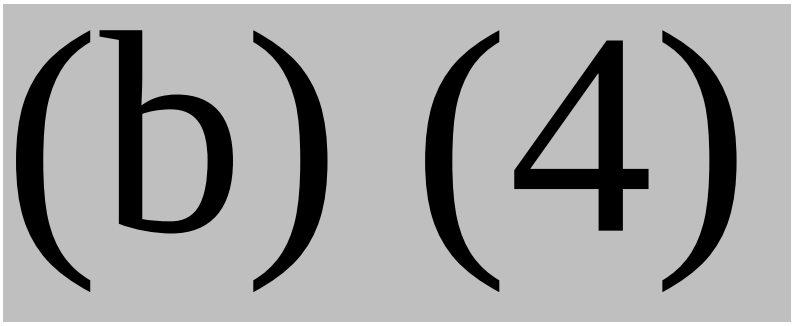
C.4.3 Heavy Metals – Finished product Astasana™ 5% CWS/S-TG

The following specifications for heavy metals are in place for Astasana™ 5% CWS/S-TG:



The procedures are based on relevant Quality and/or Good Manufacturing Practice (GMP) standards including Hazard Analysis and Critical Control Points (HACCP).

C.5 Manufacturing Process Description- Formulated Product: Astasana™ 5% CWS/S-TG - Confidential

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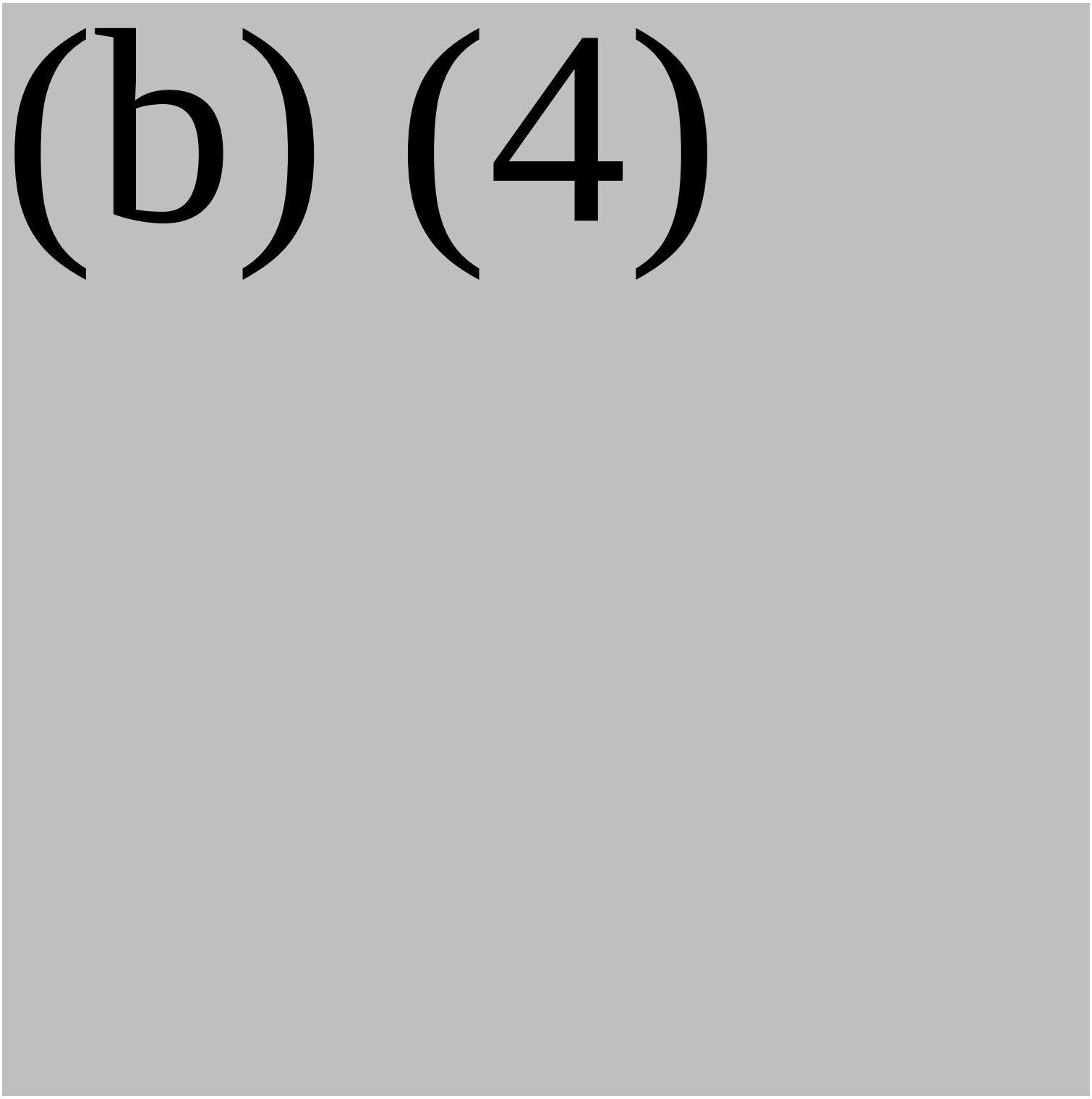
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Table 6 (b) (4)

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E. Stability- Finished Product – Astasana™ 5% CWS/S-TG

The product may be stored for 36 months (projected) from the date of manufacture in the unopened original container and at a temperature below 15 C. The 'best use before' date will be printed on the product label. Keep container tightly closed. Once opened, use contents quickly.

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F. Safety

F.1 Absorption, Distribution, Metabolism, and Excretion (ADME)

The absorption, distribution, metabolism, and excretion (ADME) of astaxanthin has been previously reviewed (GRAS notice GRN No. 294). In summary, the main elements of astaxanthin ADME in experimental animals and humans after oral exposure are as follows:

- Digestion and absorption follows a pathway similar to that dietary fat. Astaxanthin is incorporated into micelles in the small intestine, which enables diffusion across the mucosa into enterocytes, followed by incorporation into chylomicrons. Astaxanthin-containing chylomicrons enter the circulation *via* the thoracic duct and are modified by lipoprotein lipase within the vascular epithelium, until they become small enough to pass through the endothelium of the liver;

- Astaxanthin is absorbed as the free form, and esterified astaxanthin must first be hydrolyzed in the stomach. AstaSana™ is commercially available in the free form, whereas commercially available forms of astaxanthin derived from *H. pluvialis* are esterified;

- Absorption is enhanced by the presence of dietary fats (*e.g.*, olive, corn, and palm oils) and the synthetic surfactant polysorbate 80 (Clark *et al.*, 2000; Odenberg *et al.*, 2003);

- Astaxanthin is distributed in plasma lipoproteins. The proportion of astaxanthin in lipoproteins following oral administration of 100 mg to human volunteers was: 36-64% in VLDL/chylomicrons; 28-29% in LDL; and 21-23.5% in HDL (Osterlie *et al.*, 2000; Coral- Hinostroza *et al.*, 2004);

- Both rats and humans convert astaxanthin to ionol/ionone forms through oxidation of the unsaturated alkene chain at C9 (Kistler *et al.* 2000);

- Oxidative metabolism is not *via* cytochrome P450 (CYP) activity, although some CYP induction has been reported (reviewed by Lockwood & Gross, 2005); and

- Metabolites of astaxanthin are conjugated mainly into glucuronides and sulfates prior to elimination (Lockwood & Gross, 2005).

The bioavailability of astaxanthin stereoisomers in fish and humans has been examined in various studies (Schiedt *et al.*, 1981; Bjerkeng *et al.*, 1997; Osterlie *et al.*, 2000; Coral-Hinostroza, 2004; Moretti *et al.*, 2006; Rufer *et al.*, 2008). In general, the isomeric distribution in the flesh of salmonid fish reflects astaxanthin isomers in the feed. In humans, Rufer *et al.* (2008) reported that the isomer pattern in human plasma after consumption of salmon (250 g/day of wild or aquacultured salmon for 4 weeks) was similar to that of the flesh of the ingested salmon. However, compared to the isomeric distribution in the salmon flesh ingested, the relative proportion of the (3R,3'R) isomer was significantly lower, and the (3S,3'S) isomer higher, in human plasma at all time points (days 3, 6, 10, and 14), except for day 28. The authors suggested that the lack of a difference at day 28 might indicate that higher astaxanthin intakes and/or duration of exposure are required to reach a steady state of absorption and/or isomerization.

In reference to geometric isomers, U.S. FDA indicated in its review of the astaxanthin color additive petition CAP 7C0211 that:

> *"… astaxanthin can exist as different geometric isomers, known as cis- or trans-isomers. In the cis configuration, the largest functional groups on either end of a double bond are on the same side of the molecule. In the trans configuration, they are on the opposite sides of the molecule[5]. In the case of astaxanthin, there are nine double bonds that can have cis or trans configurations to give a bent or nearly linear molecular geometry. These isomers can be easily interconverted to give an equilibrium mixture. The requested specification for the cis- astaxanthin level is unnecessary because no safety concerns have been demonstrated with*

[5] Similar to fatty acids.

regard to the proportions of the cis and trans isomers of astaxanthin[6]. Furthermore, the isomeric forms are readily interconverted (Schiedt et al., 1981), occur in wild salmon, and color the flesh of salmonids. There is no evidence to suggest that the ratio of isomeric forms would affect the safety of astaxanthin. The proportion of cis/trans isomers of astaxanthin in the petitioned color additive lies within the range of the ratios found in astaxanthin extracted from the flesh of wild salmon (Turujman, 1992). Therefore, the regulation… does not contain a specification for the amount of cis-astaxanthin." (60 FR 18736, April 13, 1995)

Originally DSM has undertook an independent evaluation of information supporting the use of chemically-synthesized astaxanthin (AstaSana™) in select foods for the general population as generally recognized as safe (GRAS) through scientific procedures, which would make such use exempt from the U.S. premarket approval requirements. Astaxanthin is a carotenoid pigment with antioxidant activity. The uses of astaxanthin proposed by DSM were identical to those described in GRAS notice GRN. 294 submitted by Fuji Chemical Industry Co., Ltd, which generated no questions from U.S. FDA.

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- *The nature of astaxanthin as a pigment that is naturally present at low concentrations in the human diet;*
- *Multiple reviews of the safety data by authoritative bodies in the U.S. and Europe as part of color/feed additive petitions that considered indirect astaxanthin exposure in humans from consumption of flesh of food-producing animals (fish) fed astaxanthin. Most recently, EFSA's opinion (EFSA, 2014b) establishing an acceptable daily intake (ADI) of 14mg/day.*

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Having been the subject of multiple premarket approval applications, astaxanthin (synthesized chemically or extracted from algae/yeasts) has a considerable preclinical safety data-base.

F.2 Study Summaries

It is not practical to discuss all studies, only a subset of the most relevant studies is summarized below in Tables 4-1 through 4-4; more detailed summaries for some of the studies are provided are presented in the following section. The summary tables include the following:

Table 4-1 Summary of genotoxicity studies of astaxanthin (Edwards, et *al*.)

Table 4-2 Summary of acute, subacute, and subchronic toxicity studies of astaxanthin arranged by duration (Buesen, et *al*, Vega, et *al*.)

Table 4-3 Summary of long-term studies of astaxanthin arranged by duration (Edward, et *al*)

Table 4-4 Summary of reproductive and developmental toxicity studies of astaxanthin (Vega et *al*)

(1) Studies from DSM's files (DSM Study Reports) of chemically-synthesized astaxanthin, which were summarized in GRN No. 294 and/or various documents and scientific opinions supporting color/feed additive petitions in the United States and Europe, and whose details have been officially published in peer-reviewed scientific journals.

(2) Dozens of published human studies with several hundred subjects receiving between 1 and 40 mg astaxanthin per person per day for up to 12 months, with no evidence of any adverse effects.

Table 4-1 Summary of genotoxicity studies of astaxanthin

Test system	Test material	Final astaxanthin exposure	Conclusion	Reference
Bacterial reverse mutation assay (*in vitro*) *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and *Escherichia coli* (WP2uvrA) +/- S9	Chemically-synthesized astaxanthin	≤ 1000 µg/plate	Not **mutagenic.**	Edwards *et al.* (published)
In vitro micronucleus assay Cultured human peripheral lymphocytes	Chemically-synthesized astaxanthin	10, 33, and 100 µg/mL	Not **clastogenic.**	Edwards *et al.* (published)

[a] Identified in GRN No. 294 as "Roche, 1987," an excerpt of Color Additive Petition 7C0211 submitted to U.S. FDA by F. Hoffman-La Roche Ltd. These studies now belong to DSM Nutritional Products Ltd.

4-1.1- Bacterial reverse mutation assay (Edwards et al.)

The reverse bacterial mutation assay (Ames test) with crystalline astaxanthin was performed according to OECD Guideline for the Testing of Chemicals No. 471 and in compliance with the OECD Principles of Good Laboratory Practice (GLP). The test was performed in two independent experiments in the presence and absence of metabolic activation according to the plate incorporation method. Salmonella typhimurium strains TA1535, TA1537, TA98, and TA100 and Escherichia coli strain WP2uvrA (all obtained from Trinova Biochem GmbH, Münster, Germany) were cultured in nutrient broth and incubated in a shaking incubator. S. typhimurium strains were checked routinely for appropriate characteristics, namely histidine-requirement, crystal violet sensitivity, ampicillin resistance (TA98 and TA100), UV-sensitivity and the number of spontaneous revertants; E. coli WP2uvrA was regularly checked on tryptophan-requirement, UV sensitivity and the number of spontaneous revertants. The vehicle DMSO was used as negative control; positive control substances were sodium azide, ICR-191, 2-nitrofluorene, methylmethanesulfonate, and 4-nitroquinoline N-oxide. For metabolic activation, rat liver microsomal enzyme fraction (S9) from phenobarbital and ß-naphthoflavone induced Wistar rats (obtained from Charles River, Sulzfeld, Germany) was used.

Top agar (3 mL, containing 0.6% bacteriological agar and 0.5% sodium chloride) sterilized in glass test tubes was heated to 45° C. 0.1 mL of a fresh bacterial culture (10^9 cells/mL) of one of the tester strains, 0.1 mL of a dilution of the test substance in DMSO or the vehicle DMSO itself and either 0.5 mL S9-mix (in case of activation assays) or 0.5 mL 0.1 M phosphate buffer (in case of non-activation assays) were added. The ingredients were thoroughly mixed and the content of the top agar tube was poured onto a selective agar plate. After solidification of the top agar, the plates were incubated in the dark at 37.0 ± 2.0 °C for 48 h. The numbers of revertant colonies were counted and plates were checked for background lawn growth and precipitation.

The test was considered positive (mutagenic) when the total number of revertants exceeds two times the concurrent control in TA100 and three times the concurrent control in strains TA1535, TA1537, TA98 or WP2uvrA. Additionally, the positive response observed in one experiment should be reproducible in at least on independently repeated experiment.

In the first experiment range finding tests with eight concentrations, ranging from 3 to 5000 µg/plate, were conducted with TA100 and WP2uvrA. Subsequent experiments were

performed with five concentrations, the highest based on the amount of precipitate in the plates.

For dose-range finding, astaxanthin was tested in strains TA100 and WP2uvrA with concentrations of 3, 10, 33, 100, 333, 1000, 3330 and 5000 µg/plate in the absence and presence of 5% S9-mix.

Results of this dose range finding test were reported as a part of the first experiment (Table 4-1.1-1). Precipitation of astaxanthin on the plates was observed at the start of the incubation period at concentrations of 3330 and 5000 µg/plate and at 1000 µg/plate and above at the end of the incubation period. No increase in the number of revertants was observed upon treatment with astaxanthin in the dose range finding test with TA100 and WP2uvrA up to the highest tested concentration. Based on the observed precipitation in the dose range finding test, astaxanthin was tested up to concentrations of 1000 µg/plate in the absence and presence of 5% and 10% S9-mix in experiments 1 and 2, respectively (Tables 4-1.1-1 and 4-1.1-2). In some of the plates, slight precipitation of the test item was noted in both experiments also at 333 µg/plate at the end of the incubation period.

In both experiments, neither a reduction of the bacterial background lawn nor a decrease in the number of revertants at any tester strain was observed.

In both experiments, no biologically relevant increase in the number of revertants was observed upon treatment with astaxanthin under all conditions tested.

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(b) (4)

Table 4-1.1-2 Mutagenic response of astaxanthin in the S. typhimurium/E. coli reverse mutation assay (experiment 2).

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4-1.2 Micronucleus test (Edwards et al.)

The in vitro micronucleus test with crystalline astaxanthin was performed according to OECD Guideline for the Testing of Chemicals No. 487 (adopted 22 July 2010) and in compliance with the OECD Principles of GLP. The test was performed in cultured peripheral human lymphocytes from healthy adult, non-smoking, male volunteers aged <35 years. Blood samples were collected by venipuncture using the Venoject multiple sample blood collecting system into sodium heparin containing sterile containers. Lymphocytes were cultured for 46 ± 2 h by addition of 0.4 mL whole blood to 5 mL RPMI 1640 medium supplemented with 20% heatinactivated fetal calf serum, L-glutamine (2 mM), penicillin/streptomycin (50 U/mL and 50 µg/mL, respectively), 30 U/mL heparin, and 0.18 mg/mL phytohemagglutinin. Incubations were carried out at 37.0 ± 1.0 °C, 80-100% rel. Humidity, with 5.0 ± 0.5% CO_2 in air. For metabolic activation, rat liver microsomal enzyme fraction (S9) from phenobarbital and ß-naphthoflavone induced Wistar rats (obtained from Charles River, Sulzfeld, Germany) was used. The vehicle DMSO was used as negative control. Positive control substances, dissolved in Hanks' Balanced Salt Solution were Mitomycin C and Colchicine for experiments without metabolic activation and Cyclophosphamide for experiments with metabolic activation.

Lymphocytes were incubated with the test item for 3 h in the presence and absence of S9-mix or for 24 h in the absence of S9-mix. Cytochalasin B was added to the cells simultaneously with the test substance at the 24 h exposure time. After the 3 h incubation cells were collected by centrifugation (5 min, 365 g), washed with HBSS, resuspended in 5mL culture medium with Cytochalasin B (5 µg/mL) and incubated for another 24 h (1.5 times normal cell cycle). To harvest the cells, cell cultures were centrifuged and cells swollen by a 5 min treatment with hypotonic 0.56% (w/v) potassium chloride solution at 37 °C. Thereafter, cells were collected and fixed carefully with methanol: acetic acid fixative (3:1 v/v). Fixed cells were dropped onto cleaned and labelled microscope slides (which were blinded later) and allowed to dry before they were stained for 10-30 min with an aqueous 5% (v/v) Giemsa solution. At least two slides per culture were prepared. The dry slides were automatically embedded in a 1:10 mixture of xylene/pertex and cover-slipped. At least two slides per culture were prepared.

Cytotoxicity in the lymphocyte cultures was determined using the cytokinesis-block proliferation index (CBPI index).

At least 1000 binucleated cells per culture were examined by light microscopy for micronuclei. In addition, 1000 mononucleated cells per culture were scored for micronuclei separately. Multinucleated cells and cells undergoing apoptosis were not scored. Criteria for scoring micronuclei were adapted from Fenech (1996): For inclusion, diameter of the micronuclei had to be less than one-third the area of the main nucleus, micronuclei had to show similar staining characteristics as the main nucleus, and micronuclei should be separate from main nucleus. Results of treatment groups were compared with the corresponding control values by means of a Chi-square test; results were considered significantly different from control at $p < 0.05$.

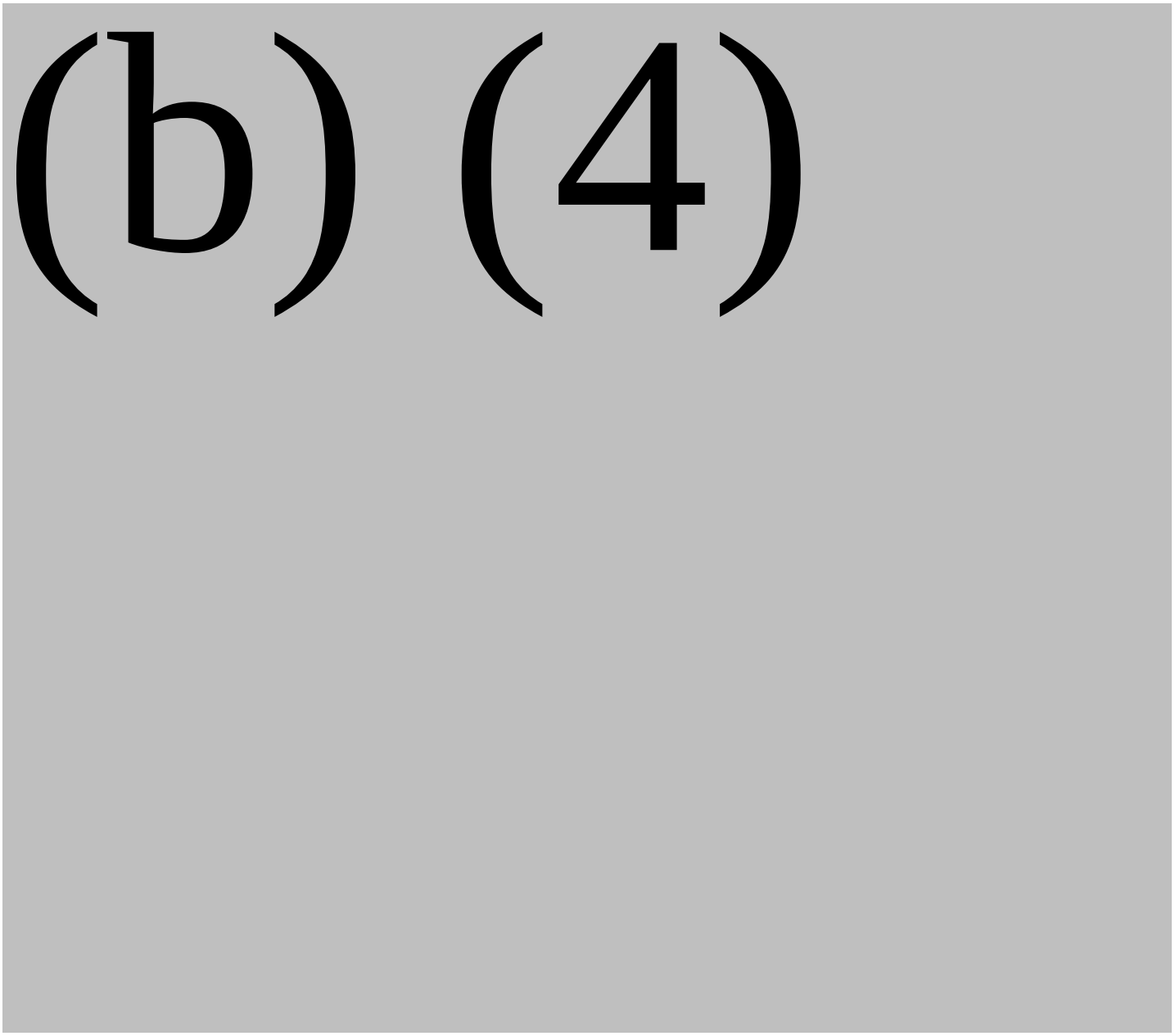
Three analyzable concentrations were scored for micronuclei. The highest tested concentration was determined by the solubility of astaxanthin in the culture medium at the 3 h exposure time.

In the dose-range finding test, astaxanthin precipitated in the culture medium at a concentration of 333 µg/mL.

In the first assay with 3 h exposure, concentrations of 10, 33, and 100 µg/mL were selected. At 100 µg/mL, precipitation of astaxanthin was noted. Astaxanthin did not induce a statistically significant or biologically relevant increase in the number of mono- and binucleated cells with micronuclei in the absence and presence of S9-mix (4-1.2-1).

For the second experiment with 24 h incubation time without metabolic activation, doses of 33, 100, and 333 µg/mL astaxanthin were selected. Precipitation of astaxanthin was noted at 100 and 333 µg/mL. The test item induced a non-concentration-dependent and statistically non-significant increase in micronuclei in binucleated cells and a significant increase of micronuclei in mononucleated cells (data not shown). However, the increase observed in mononucleated cells was within the acceptability range of the test. Since the number of micronucleated cells in this cytogenetic assay was relatively high throughout all groups (including solvent control), the experiment was repeated to verify the results. In the repeat experiment (Table 4-1.2-2) astaxanthin did not induce a statistically significant or biologically relevant increase in the number of mono- and binucleated cells with micronuclei after 24 h incubation time.

In both experiments, the respective positive controls induced statistically significant increases in the number of micronuclei in binucleated (mitomycin C, cyclophosphamide) and mononucleated cells (colchicine), indicating the validity of the assay.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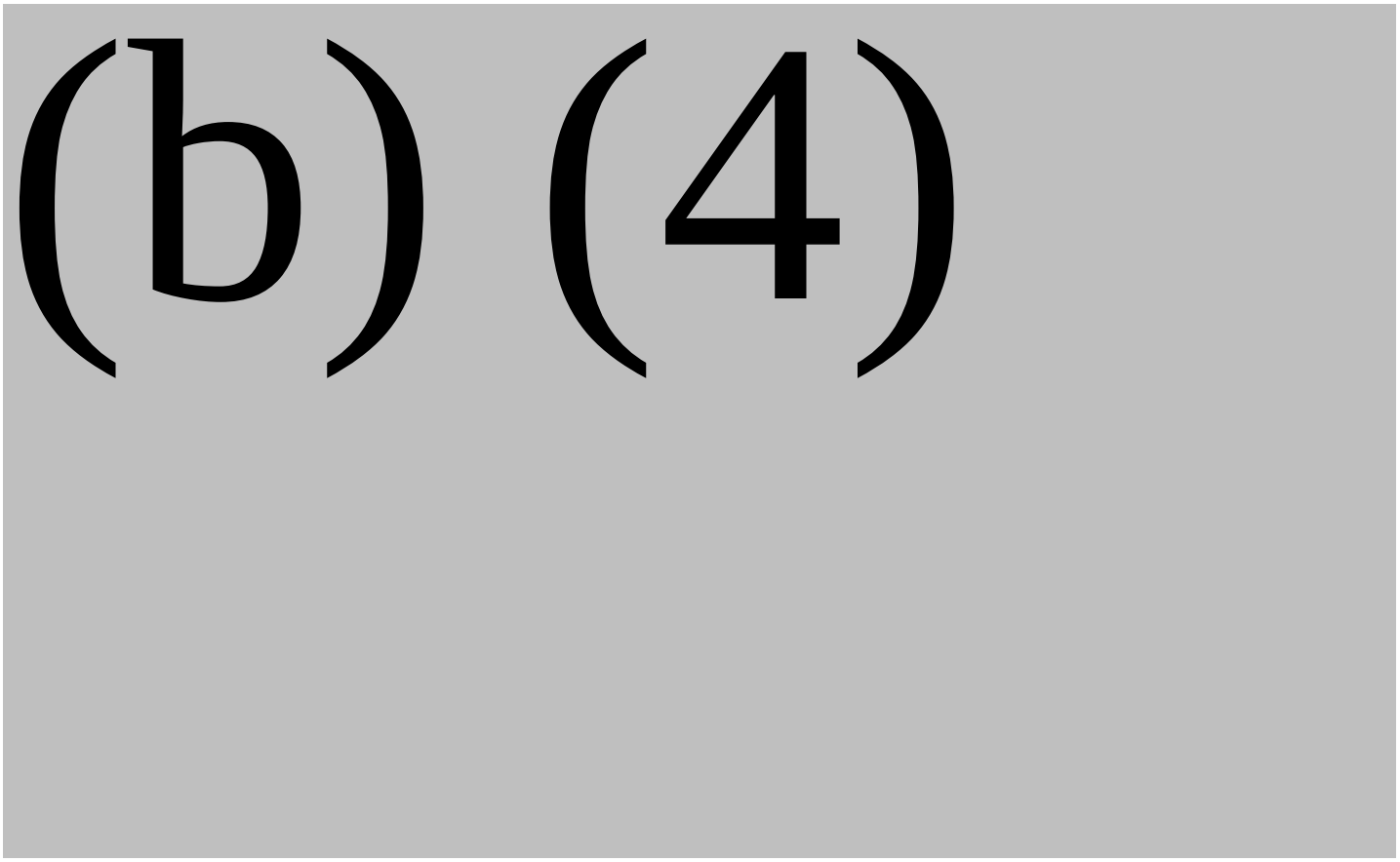
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Table 4-2 Summary of acute, subacute, and subchronic toxicity studies of astaxanthin

Duration	Species	Route	Test material	Final astaxanthin exposure	Conclusion	Reference
13 weeks	Rat (Wistar)	Oral, diet	Chemically-synthesized astaxanthin	Mean for males: 70.94, 216.48, 701.76 mg/kg/day Mean for females: 79.8, 262.44, 922.58 mg/kg/day	NOAEL: 700 mg/kg/day (male) NOAEL: 920 mg/kg/day (female)	Buesen *et al.* (2015) (published)
13 weeks	Rat (HanIbm Wistar)	Oral, diet	Chemically-synthesized astaxanthin	0, 256 (range: 220-310), 513 (range: 430-620), 1033 (range: 880-1240) mg/kg bw/day	NOAEL: 1033 mg/kg bw/day	Vega *et al.* (published)[a]

[a] Identified in GRN No. 294 as "Roche, 1987," an excerpt of Color Additive Petition 7C0211 submitted to U.S. FDA by F. Hoffman-La Roche Ltd. These studies now belong to DSM Nutritional Products Ltd.

4-2.1 13-Week study in rats (Vega et al.)

(b) (4)

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(b) (4)

4-2.2 13-Week study in rats (Buesen et al., 2015)

(b) (4)

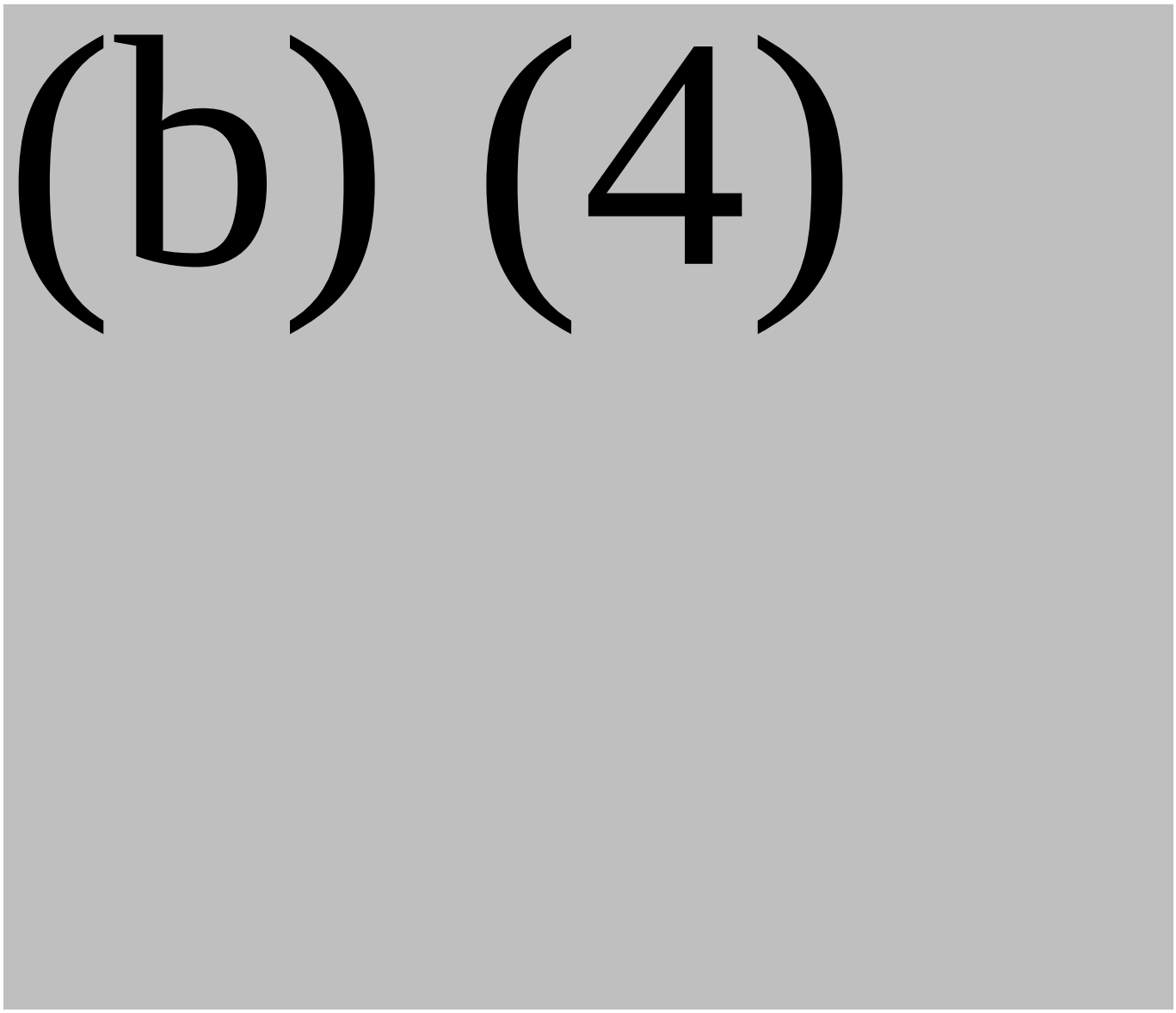
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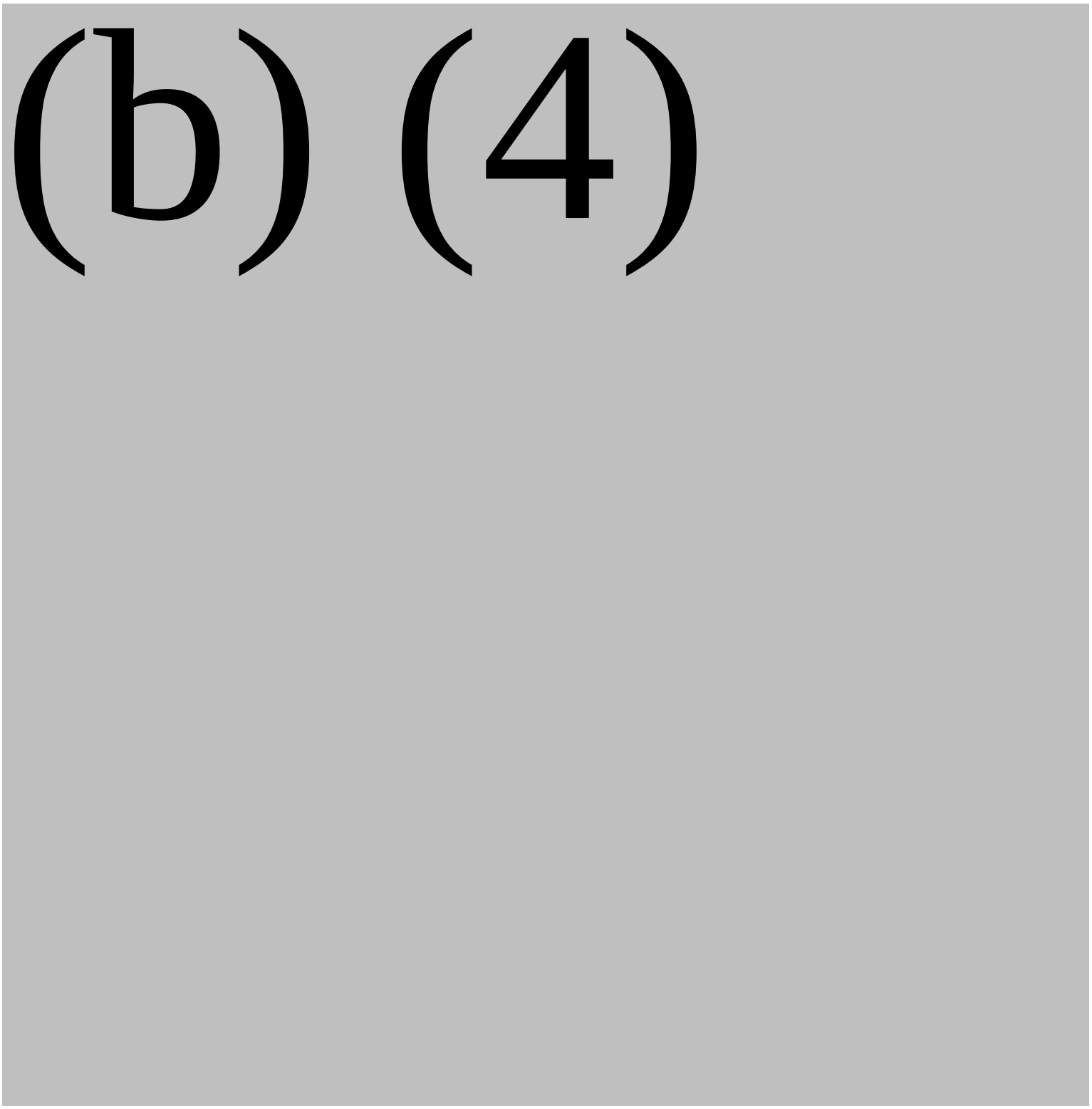
Table 4-3 Summary of long-term studies of astaxanthin arranged by duration

Duration	Species	Route	Test material	Final astaxanthin exposure	Conclusion	Reference
Chronic Toxicity						
(b) (4)						
						Confidential
Carcinogenicity						
2 years	Rat	Oral, diet	Chemically-synthesized astaxanthin	0, 40, 200, 1000 mg/kg bw/day	NOAEL: 40 mg/kg bw/day*	Edwards *et al.* (published)

* No-effect level for benign neoplatic change.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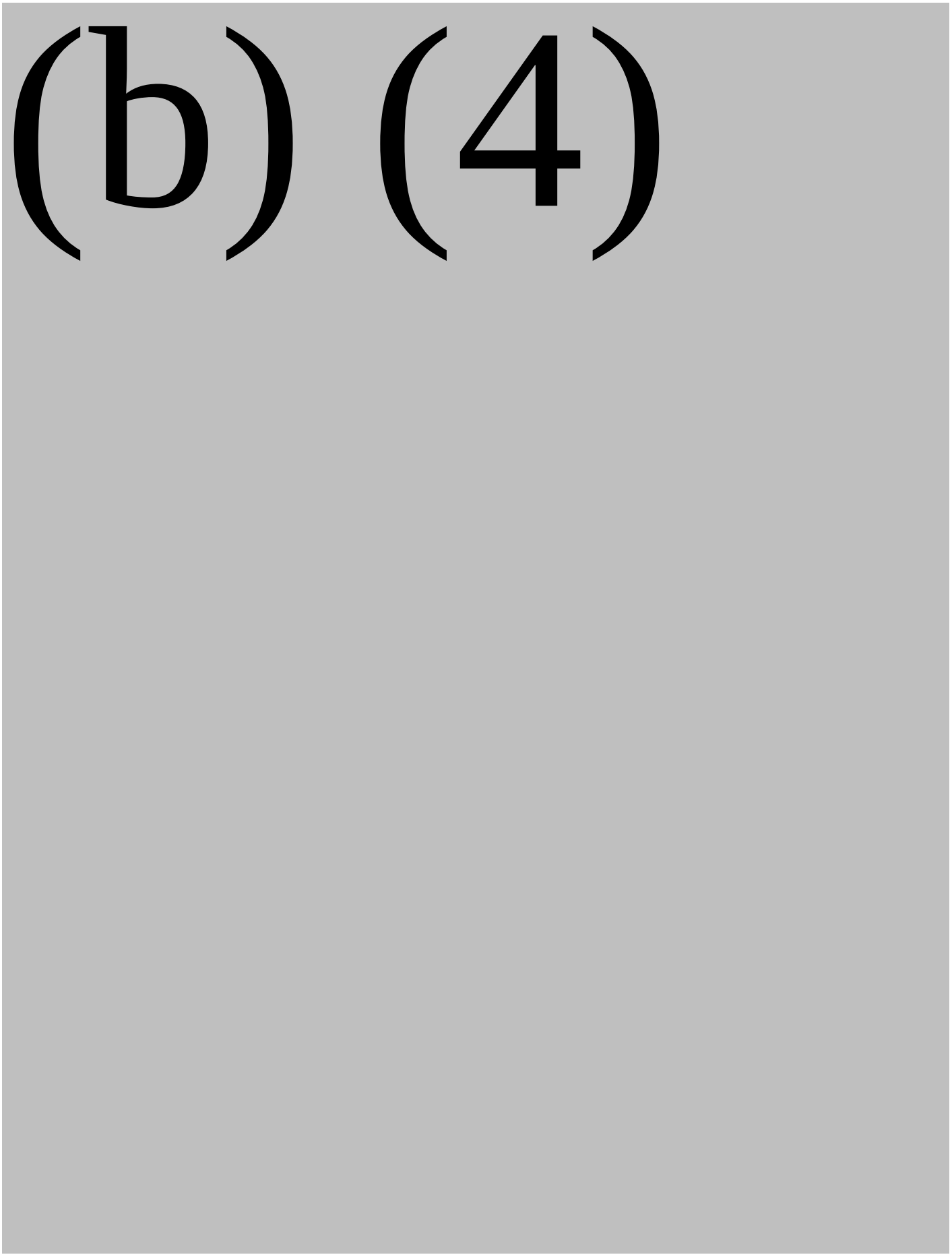
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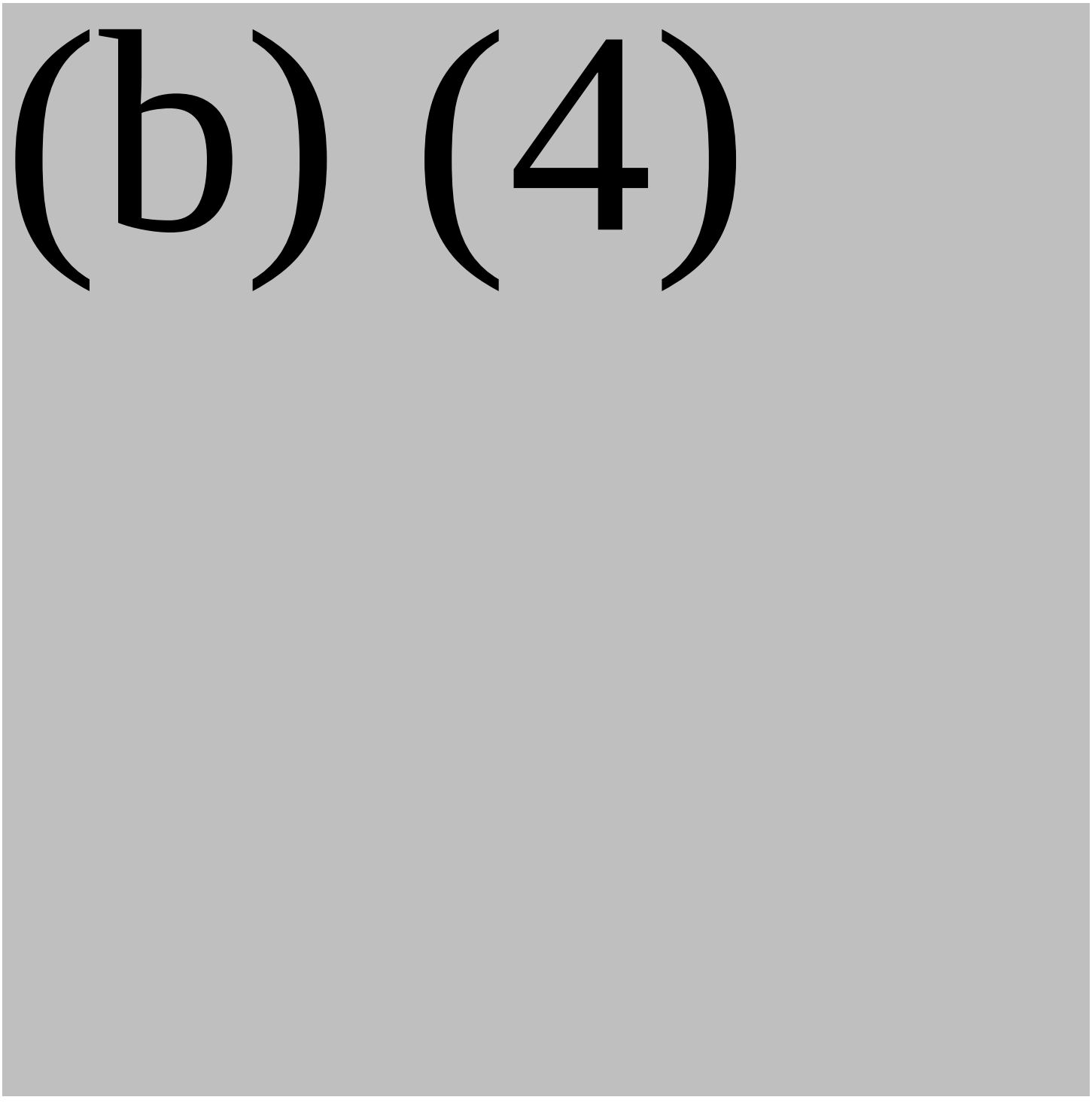
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4-3.2 B. 2-Year carcinogenicity study in rats (Edwards et al.,)

Wistar rats (60/sex/group) received standard feed (control), feed containing placebo beadlets (placebo control), or feed containing astaxanthin (8%) beadlets; the latter was intended to provide 40, 200, and 1000 mg astaxanthin/kg bw/day. After 1 year of treatment, subsets of animals (10/sex/group) from the placebo control and astaxanthin groups were selected for a 1- year recovery period and treatment was discontinued. The remaining animals continued treatment for another year.

Survival after the 2-year treatment period was between 76% and 88% for males and between 56% and 82% for females. In males, the mortality rate was similar in all groups, whereas in females the mortality rate was lower at the high dose, when compared to controls. Survival of the recovery animals was between 80% and 100% for males and between 70% and 100% for females, without significant difference between controls and treated groups.

There were no treatment-related clinical signs, except for a dark red discoloration of the feces at 200 and 1000 mg/kg bw/day, which was considered related to the elimination of unabsorbed test item. In the recovery groups, the discoloration disappeared within one week after discontinuation of treatment. Fecal discoloration has been observed in several other repeat-dose studies with astaxanthin.

As was observed in a 1-year study in rats, administration of beadlets (placebo and astaxanthin) in this study was associated with reduced food consumption (by 11-16%), compared to animals receiving the standard rodent feed. Improved food consumption was observed in recovery group animals that were withdrawn from treatment.

Body weight gains of animals receiving the dietary admixtures of placebo formulation with or without astaxanthin were lower over the 2-year treatment period when compared with the untreated controls. The total weight gain was 17-19% lower in males, and 33-38% lower in females. In females, at 200 and 1000 mg/kg bw/day, total weight gains were lower as compared to the weight gains of the placebo control. Body weight compensation was observed in the recovery groups over the second year of the study.

After 1 year of treatment, astaxanthin plasma levels were approximately twice as high in

females as in males, whereas after 2 years of treatment, the plasma levels of males had increased and were almost comparable to the levels in females. Although there was high variability in individual plasma levels, the group means showed a non-linear relationship to dose. No astaxanthin was detected in the plasma of recovery group animals after the 1-year recovery period.

Clinical laboratory parameters showed no substantial differences between the untreated control and placebo control groups. A number of slight but statistically significant differences in hematological parameters were observed at the high astaxanthin concentration. Changes in red blood cell parameters, *e.g.*, lower numbers of red blood cells (RBC) and lower packed cell volume (PCV) or higher mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), were mostly seen at the end of the study. After 2 years of astaxanthin treatment at 200 mg/kg bw/day and 1000 mg/kg bw/day, RBC number were lower in both males and females. Females also had elevated calculated indices for MCH and MCHC. However, none of these values exceeded the normal physiological range. Variations in the white blood cell (WBC) profile were occasionally seen during the study, but with no substantial alteration of the total number of white blood cells. No relevant hematological changes were observed in the recovery animals after 1 year of exposure followed by a 1-year recovery period.

A number of slight but statistically significant biochemical changes were observed in both males and females at 200 mg and 1000 mg/kg bw/day. Cholesterol levels were significantly higher in both sexes after 1 year of treatment, and in females after 2 years of treatment. These values did not exceed the expected physiological norm. Higher bilirubin levels and higher alkaline phosphatase (AP) activity in females after 1 and 2 years of treatment included individual values at the upper limit or exceeded the expected norm. Slightly higher aspartate aminotransferase (AST) activity in 1000 mg/kg bw/day females was observed after 2 years. These changes in bilirubin, AP and AST might have been related to liver toxicity observed in female rats (see below). No relevant biochemical changes were observed in the recovery animals after the second year of the study.

There was no difference in the type or incidence of palpable masses in male or female groups during the treatment period. Upon necropsy, a yellow-orange discoloration of the adipose

tissue was observed, which was not unexpected after prolonged ingestion of the lipophilic test item. A slightly yellow discoloration of the adipose tissue was still observed in some recovery animals after the 1-year recovery period. Furthermore, microscopic observation of pigmentation in hepatocytes, particularly in female rats, might have been due to accumulation of astaxanthin.
Gross lesions in animals that had died during the study or that were sacrificed after 2 years of treatment were comparable between controls and astaxanthin-treated groups.

Organ weight variations, *e.g.*, of the heart, brain or spleen, in the placebo or astaxanthin-treated groups were considered to be due to the lower body weights of treated groups *vs*. the untreated control. In astaxanthin-treated groups, slightly lower kidney (relative to body weight) and testis weights were observed in males at 1000 mg/kg bw/day, and lower ovary and adrenal weights were observed in females at 200 and 1000 mg/kg bw/day, when compared with the placebo control. However, histopathological examination showed no abnormal findings correlating with the organ weight changes in these groups. Upon histopathological examination, the liver was identified as the target organ and the following findings with respect to incidences and/or severities distinguished treated rats from controls.

Non-neoplastic findings:

The non-neoplastic lesions seen in the chronic one-year rat study and after two years of treatment in the carcinogenicity rat study were similar except that some lesions were more frequent and/or severe after 2 years of treatment in females at the mid- and high-astaxanthin concentrations.

In male rats, centrilobular hepatocellular vacuolation and fat deposition were considered minor effects. In female rats, increased deposition of pigment observed in the hepatocytes and macrophages might have represented accumulation of the test item. The increased incidence of hepatocellular hypertrophy (increase in cell size) observed in females of the 40, 200 and 1000 mg/kg bw/day groups was regarded as an adaptive metabolic response, rather than a toxic effect of the test item. The increased incidence of single-cell necrosis, fatty change (incl. vacuolated foci) and inflammatory cell foci were considered to reflect a probable toxic effect of the test item. The increased incidence of multinucleated hepatocytes was considered to represent a regenerative process secondary to cell injury or

cell death.

In the rats killed at termination of the 2-year treatment period, an increased incidence of biliary cysts was noted in the liver of females at 200 and 1000 mg/kg bw/day. It is not clear from this study whether or not this increase was related to the administration of the test item. After the 1- year recovery period, the findings in the liver did not distinguish treated rats from the vehicle controls, indicating the reversibility of these findings. All other non-neoplastic findings noted in this study were considered to be incidental findings commonly noted in rats of this strain and age.

Neoplastic findings:
In male rats, there was no significant increase in the incidence of tumors at any site. An increased incidence of benign hepatocellular adenomas was observed in female rats at 200 and 1000 mg/kg bw/day after 2 years; a slightly higher incidence in female rats at 40 mg/kg bw/day in comparison to the controls (5/50 treated rats *vs*. 2/50 and 1/50 in the control groups) was considered to be incidental, as the slight increase was not statistically significant and was within the control range (0/50 to 6/50) of other contemporary studies with the same rat strain.

No treatment-related neoplastic changes were found in the additional subgroups of recovery rats (10 male and 10 female rats/group) that had been treated during the first year only, and remained untreated during the second year to assess recovery. The incidence of hepatocellular carcinomas was not statistically significantly increased in the female rats. There was no significant increase in hepatocellular adenomas or carcinomas in the male rats.

The benign hepatocellular adenomas in female rats were observed in the presence of hepatotoxicity, which was more severe in females as compared to males. Major changes in females consisted of pigment accumulation, hepatocyte hypertrophy, inflammatory cell foci, single-cell necrosis, hepatocyte vacuolation, patchy hepatocellular fat deposits, and polynucleated/cytomegalic hepatic cells. Several of these findings, including polynucleated/cytomegalic hepatic cells were also observed in the control and placebo control rats, but at a lower incidence.

It was concluded that, in male rats, astaxanthin administered in the feed for 2 years was not

toxic at up to 200 mg/kg bw/day or tumorigenic at up to 1000 mg/kg bw/day. In females, a dose- related increased incidence of hepatocellular adenomas was observed at 200 and 1000 mg/kg bw/day in the presence of hepatotoxicity and subsequent regenerative processes, only at the end of the 2-year treatment period. No significant increase was seen in the incidence of hepatocellular carcinomas. It is concluded that hepatotoxicity in female rats represents a single- sex and species-specific phenomenon. Therefore, the hepatocellular adenomas are considered to be secondary to hepatotoxicity and subsequent regenerative processes. Since the effect was limited to benign hepatocellular tumors, this was not considered to represent a carcinogenic potential of astaxanthin.

Table 4-4 Summary of reproductive and developmental toxicity studies of astaxanthin

Study type	Species	Route	Test material	Final astaxanthin exposure	Conclusion	Reference
(b) (4)						
						Confidential
Developmental toxicity (gestation days 7-16)	Rat	Oral, diet	Chemically-synthesized astaxanthin	0, 250, 500 and 1000 mg/kg bw/day	NOAEL: 1000 mg/kg bw/day	Vega *et al.* (published)

[a] Identified in GRN No. 294 as "Roche, 1987," an excerpt of Color Additive Petition 7C0211 submitted to U.S. FDA by F. Hoffman-La Roche Ltd. These studies now belong to DSM Nutritional Products Ltd.

4-4.1 (b) (4)
- Confidential

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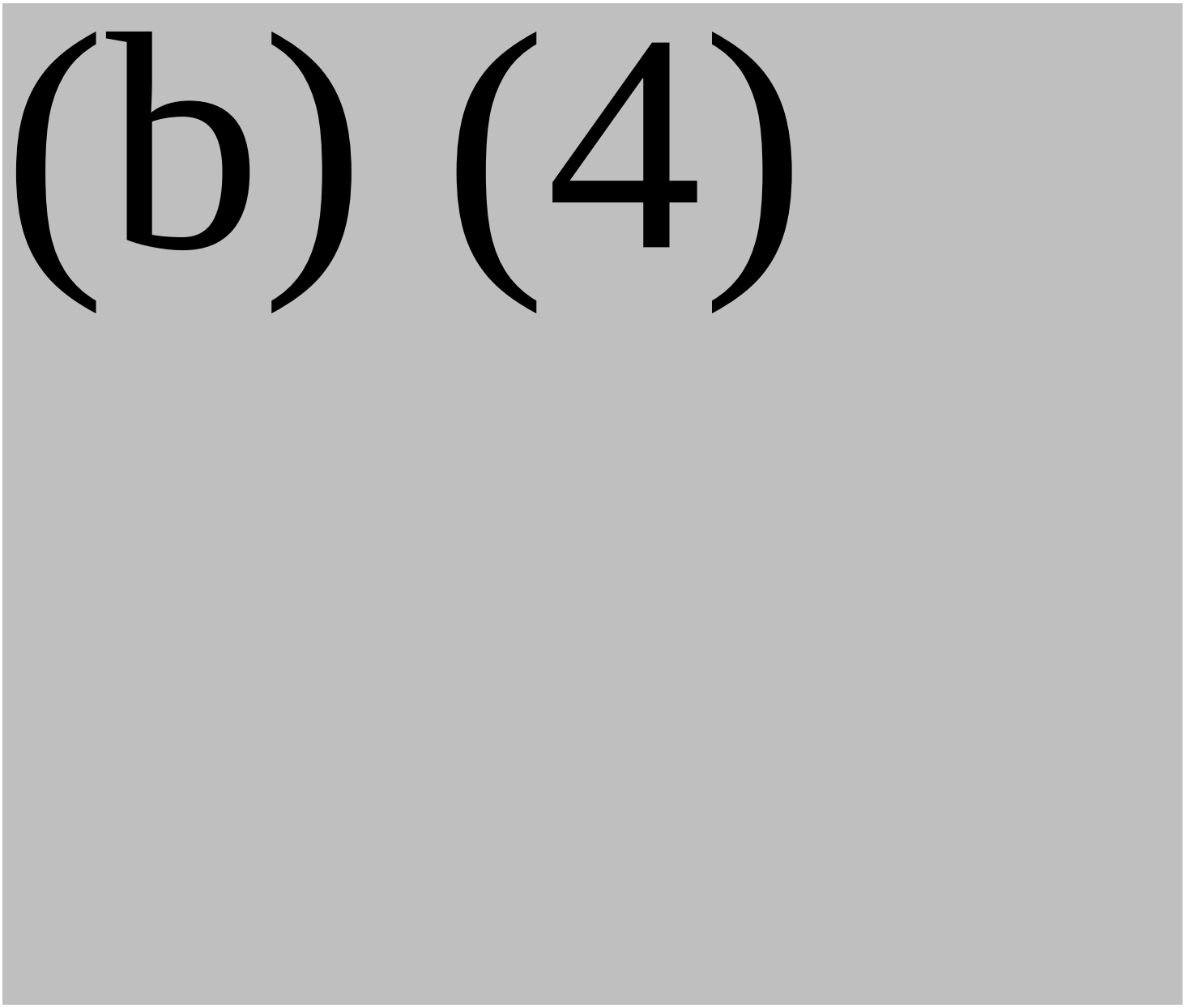
(b) (4)


(b) (4)

G. 1 Safety Information (Astaxanthin) to support Conditions of Use of DSM's Astasana™ 5% CWS/S-TG in Dietary Supplements.

Table 5 provides a summary of the expected astaxanthin consumer exposures, compared to values derived from experimental animal studies, human studies, and other sources and supports the use of Astasana™ in dietary supplements for the general population for up to 12mg/person/day.

Astaxanthin exposures in the U.S. population from the proposed food uses is expected to be within the range of what has been shown to be safe for humans based on multiple clinical investigations, the EFSA ADI, and GRAS notice GRN No. 294. The projected 90th percentile intake value of 1.25 mg/person/day is also nearly 10 times lower than the value (12 mg/person/day) derived from the lowest reported NOAEL in multiple-dose experimental animal studies, 20 mg/kg bw/day, reported by Onodera *et al.* (1997) to have had no effects on mortality or other signs of toxicity in rats following oral exposure for 13 weeks.

Table 5 Expected astaxanthin exposures *vs.* key safety endpoints (published studies)

Endpoint	mg/kg bw/day[3]	mg/person/day[3]
EFSA†† (2019) ADI 0.2 mg	**0.2**	**14.0**
Range of NOAELs from 13-week studies, adjusted[1]		
Onodera, *et al.* (1997) no toxicity level: 20 mg/kg bw/day	0.2	**12**
Vega, *et al.* NOAEL: 1033 mg/kg bw/day	10.33	**619.8**
NOAEL from rat carcinogenicity study, adjusted[2]		
Edwards, *et al.* NOAEL: 40 mg/kg bw/day	0.4	**24**
Range of exposures from human studies	0.017-0.67†	**1-40**

[1] Derived by applying a 100-fold uncertainty factor for inter- and intraspecies differences to the respective lowest and highest no toxicity/NOAEL from available 13-week studies (see Table 4-2).

[2] Derived by applying a 100-fold uncertainty factor for inter- and intraspecies differences to the NOAEL (see Table 4-3).

[3] Based on a 60-kg person.

†Actual mg/kg bw/day value was not specified (see Tables 4-5 and 4-6). Calculated value (__ mg/person/day ÷ 60 kg body weight =_____mg/kg bw/day).

††ADI is 0.2 mg AXN/kg bw/day => 70 kg adult is 14 mg/day.

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