NEW DIETARY INGREDIENT (NDI) SAFETY INFORMATION OF LEMNARED® ASTAXANTHIN CRYSTAL

Lemnaceae Fermentation Inc.

August 30, 2020

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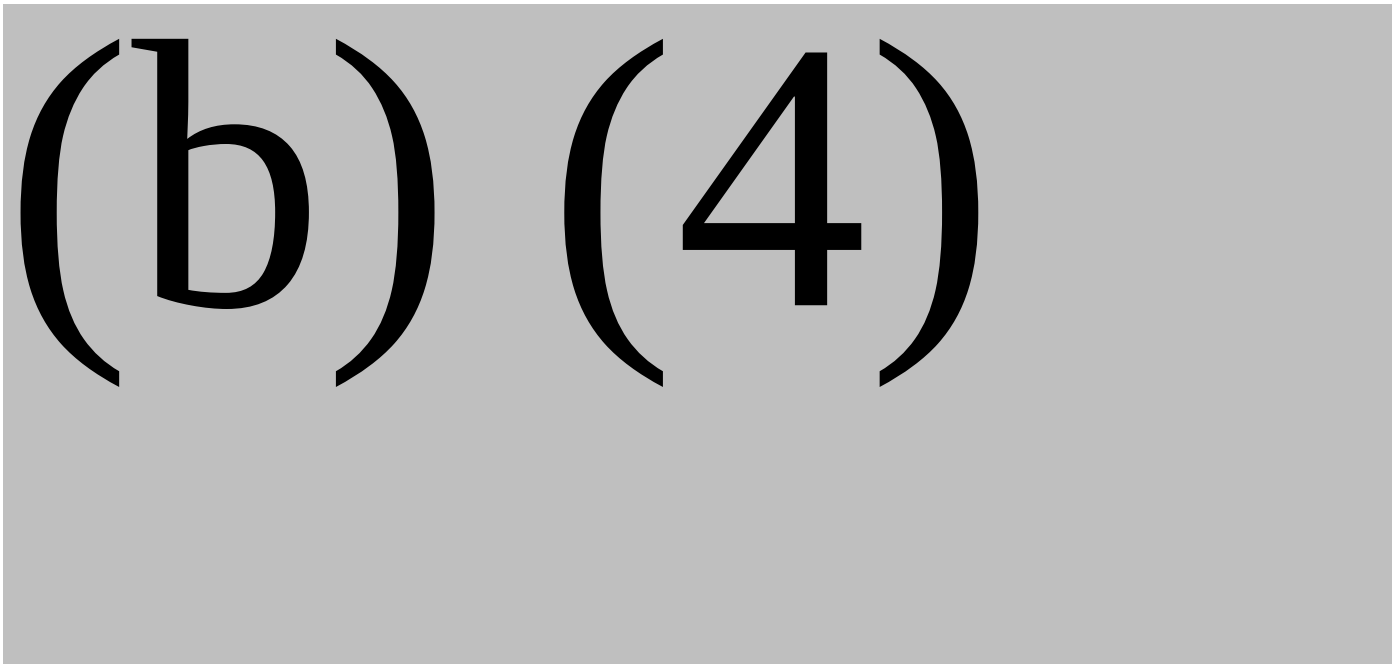

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1. New Dietary Ingredient Identity Information

1.1 Description of The Identity of The New Dietary Ingredient (NDI)

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Table 1. NDI notifications related to astaxanthin

NDI No.	Date	Title of Notification
NDI 632	01/20/2010	Puresta Oil from Yamaha Motor Co., LTD
NDI 717	06/27/2011	*Haematococcus Pluvialis* Extract Containing Astaxanthin Esters from Cyanotech Corporation.
NDI 742	12/07/2011	Astaxanthin esters from *Haematoccocus pluvilai* from Fuji Health Science.
NDI 815	12/04/2013	Astaxanthin esters from *Haematococcus Pluvialis* Calastin from Genovia Bio LLC.
NDI 829	04/09/2014	Astaxanthin-rich Carotenoid Extract [ARE] from JX Nippon Oil and Energy Corporation.
NDI 884	04/09/2015	Haematococcus pluvialis J. Von Flotow, 1844 extract containing astaxanthin esters (AstaZine) from BGG North America, Inc.
NDI 943	01/09/2016	Haematococcus Pluvialis extract containing astaxanthin esters under trade name AstaZine from BGG North America, Inc.
NDI 957	09/11/2016	AstaPure oleoresin containing 10% astaxanthin esters from Haematococcus pluvialis Flotow under trade name AstaPure from Algatechnologies.
NDI 1067	26/02/2018	Astaxanathin-rich oleoresin from Yunnan Alphy Biotech Co. Ltd.

1.2 Description of evidence verifying the identity of the NDI

The chemical composition of LemnaRed® astaxanthin crystal is analyzed by UV-VIS spectrophotometric analysis, reversed-phase high performance liquid chromatography (HPLC), and chiral HPLC analysis.

1.2.1 UV-VIS Spectrophotometric Analysis

The total carotenoid content and the identity of the LemnaRed® Astaxanthin is analyzed by UV-VIS spectrophotometric analysis. It involves measuring the maximum light absorbance of the sample EtOH solution at the wavelength of 478 nm and compare the spectrum with that of the astaxanthin standard. The UV-VIS spectrophotometric analysis result of LemnaRed® Astaxanthin is showed in

Figure **1**. It indicated that the resulting absorption spectrum of LemnaRed® Astaxanthin was complied with that of the astaxanthin standard. The detailed test method is provided in Appendix 1.

Additionally, the total carotenoid content (%) of the LemnaRed® astaxanthin crystal was calculated by using the following formula:

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1.3 Manufacturing of NDI

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1.3.1 Raw materials

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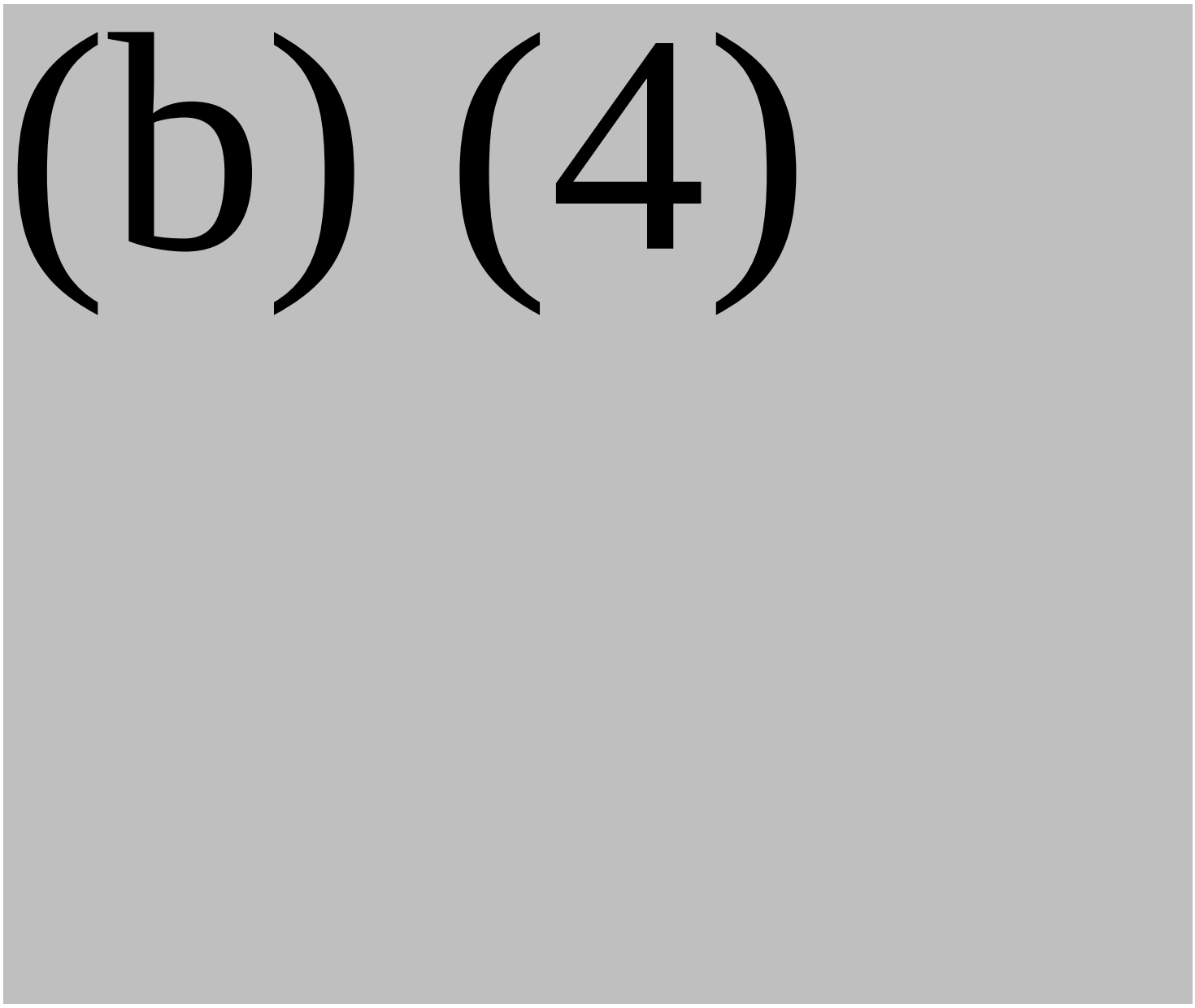
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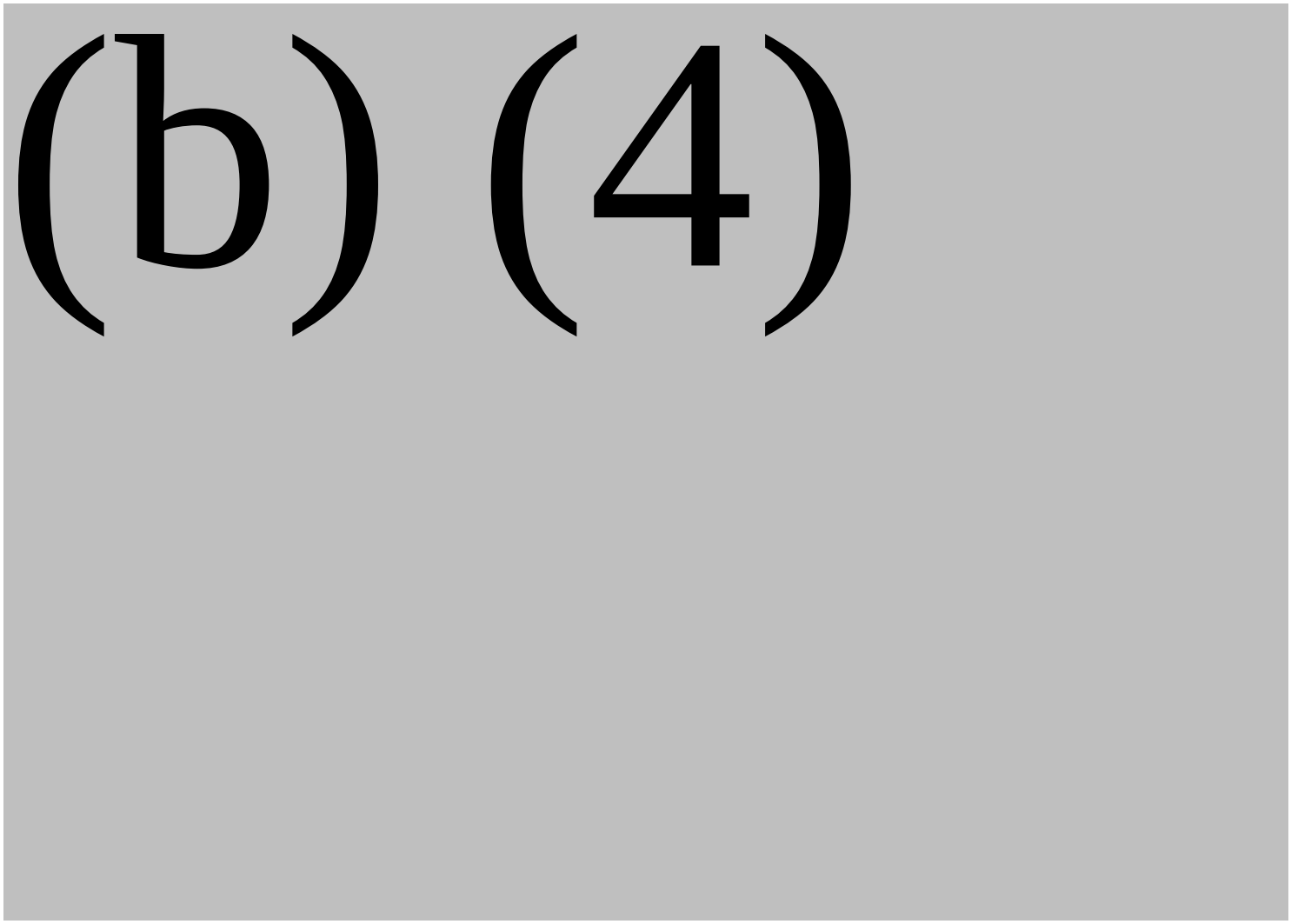
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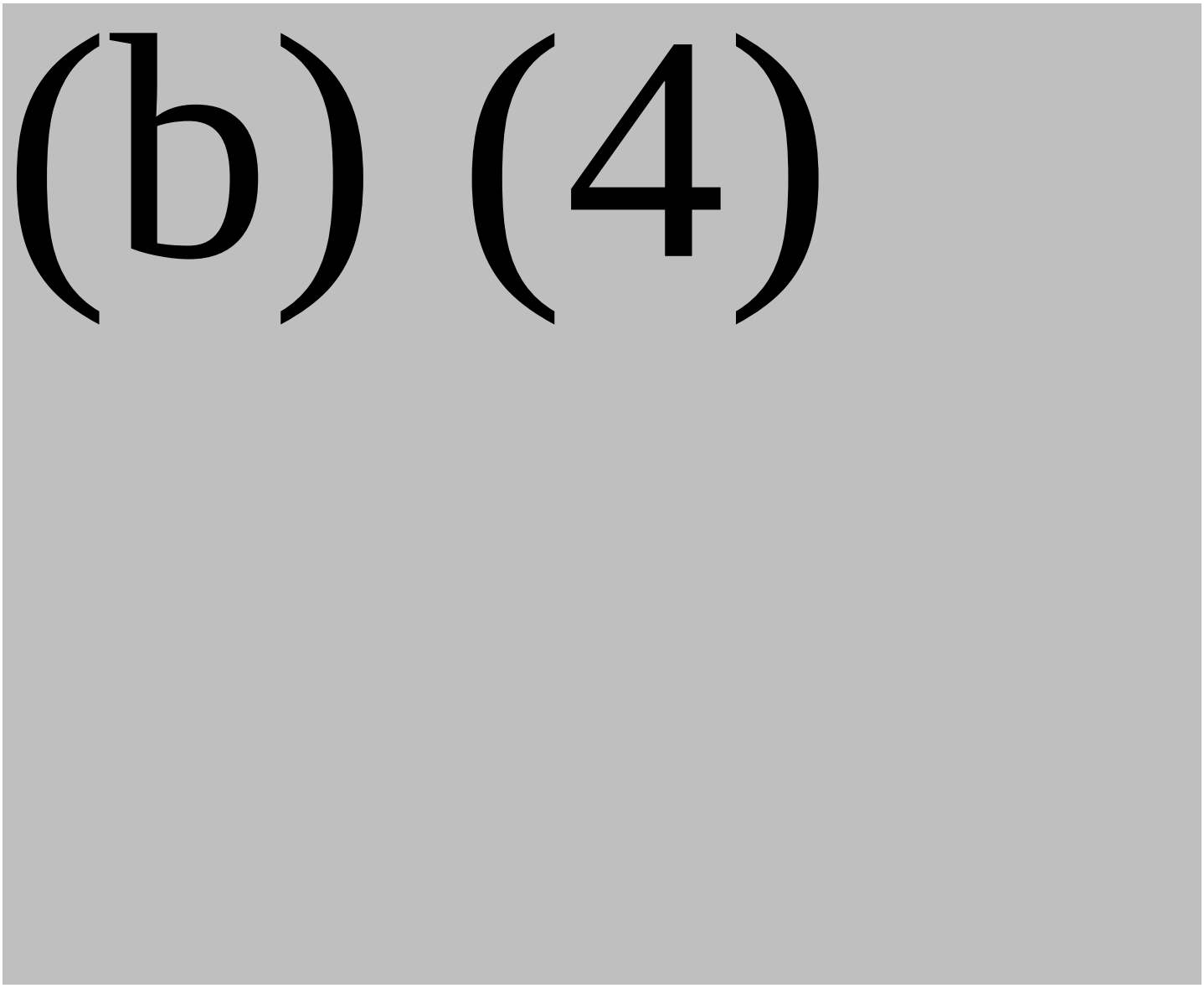
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1.3.2 Formulation ingredients

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1.3.3 Manufacturing process

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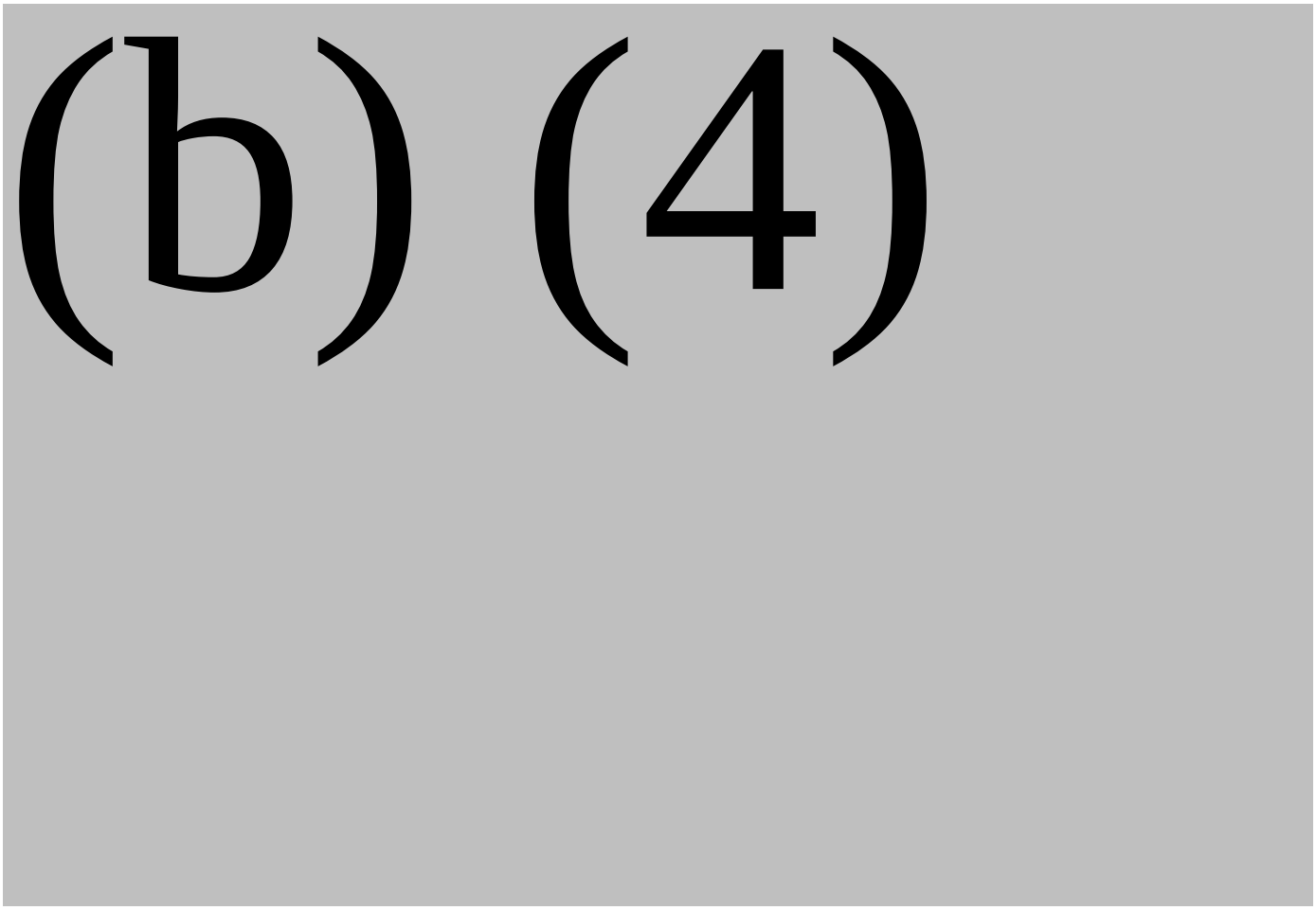
1.3.4 NDI specifications

1.3.4.1 The Specifications of LemnaRed® Astaxanthin Crystal



1.3.5 Methods of analysis

The methods used to analyze product specifications are listed in section 1.3.4, and analytical procedures are detailed in the Appendix.

1.3.6 Analysis of potentially toxic processes

All manufacturing procedures are conducted in accordance with the hazard analysis critical control points (HACCP) plan for the production of LemnaRed® astaxanthin crystal. Quality control analysis is performed on each batch according to specifications described in Section 1.3.4. The detailed analysis of potentially toxic processes is provided in Table 9.

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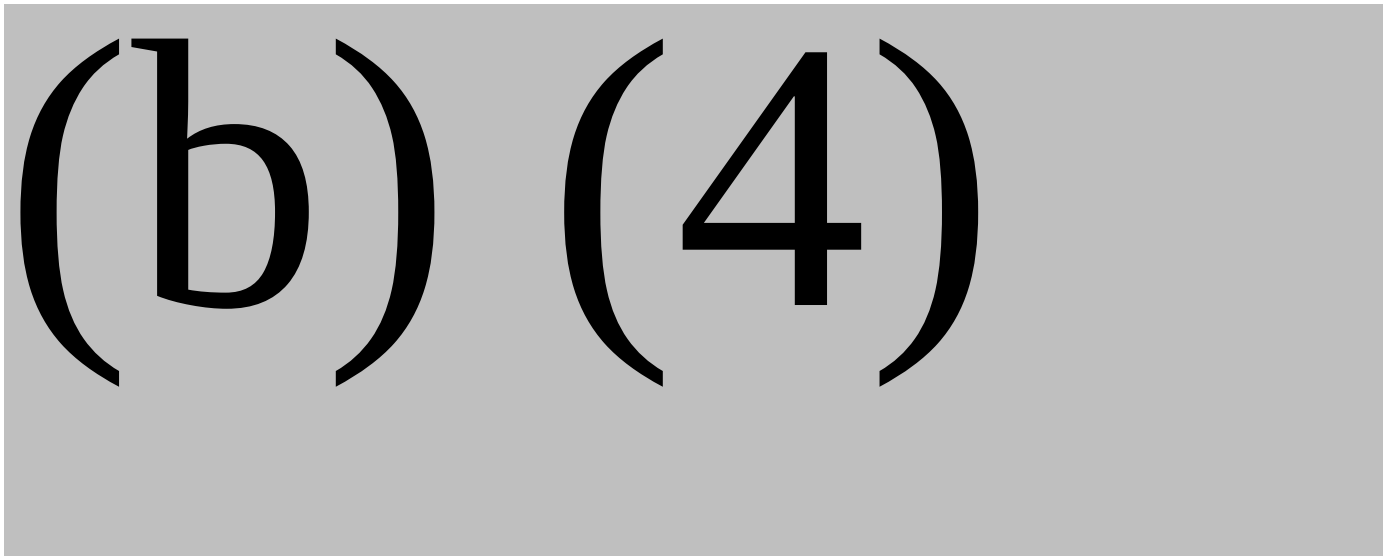

1.3.7 Disintegration and dissolution profile

LemnaRed® astaxanthin crystal is produced in powder form; therefore, disintegration and dissolution profiles are inapplicable.

1.3.8 Shelf-life and storage conditions

1.3.8.1 Shelf-life and Storage Conditions of LemnaRed® Astaxanthin Crystal

The stability of the product remains highly satisfactory over a period of at least one year from the date of manufacture, particularly when stored below -20℃ (Figure 29 and Figure 30). Stability is assessed by determining the concentration of astaxanthin via HPLC throughout the storage period.

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2. Dietary Supplement Manufacture

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2.1. Raw materials

The raw materials used in the production of LemnaRed® astaxanthin softgels include the following:

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2.2. Formulation ingredients other than the NDI

Ingredients used in the formulation of LemnaRed® astaxanthin softgels:

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2.3 Manufacturing process

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2.4. Product specifications

The Specifications of LemnaRed® Astaxanthin Softgels are provided in Table 10.

Table 10. Specifications and Analytical methods of LemnaRed® astaxanthin softgels.

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2.5. Methods of analysis

The methods used to assess the concentration and sanitary quality of astaxanthin are described in Section 2.4 and provided in Appendix.

2.6. Analysis of potentially toxic processes

The critical control points in the production of LemnaRed® softgels are when (1) the raw materials are combined and (2) metals are detected. All of the raw materials used in the production of the softgels are food grade and meet relevant quality specifications.

2.7. Disintegration and dissolution profile

Dissolution test were conducted in accordance with the procedures described in "〈2040〉 Disintegration and Dissolution of Dietary Supplements.[15]" The average disintegration time of the softgels was determined to be 7.3 mins.

2.8. Shelf-life and storage conditions

The shelf-life of LemnaRed® astaxanthin softgel is 24 months when stored at an ambient temperature of 77 to 86 degrees Fahrenheit and protected from humidity. When considering how product quality degrades over time, the primary concern for softgels is that they can lose their potency during long-term storage. (b) (4)

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3. History of Use and Other Evidence of Safety

3.1 History of use

3.1.1 History of Use of Astaxanthin

Astaxanthin is a natural red pigment found in a variety of seafood, such as salmon, trout, krill, shrimp, and crayfish, and has been consumed by human for centuries without any adverse effects has been reported. Astaxanthin levels in the muscle tissue of wild salmon range between 1 and 58 mg/kg [16] and, in commercially farmed salmon, astaxanthin levels range between 6 and 8 mg/kg. The concentration of astaxanthin in commercially farmed salmon does not usually exceed 10 mg/kg. Beyond this level, differences in concentration are not observable to the human eye [17]. A human consuming 300 to 600 g of fish flesh a day takes in 17.4 to 34.8 mg astaxanthin. Typical astaxanthin consumption varies from country to country; however, a daily intake of 17.4 to 34.8 mg astaxanthin is generally accepted as safe for humans.

Astaxanthin has been commercially available since the late 1980s. This began with chemically synthesized astaxanthin, which was approved as a food coloring for fish foods (particularly salmon and trout), by the European Commission in 1989 and by the U.S. Food and Drug Administration (FDA) in 1995. Astaxanthin extracted from *Haematococcus* algae has been used as a human dietary supplement since 1995, when the Swedish company, AstaCarotene AB, began marketing Astaxain™ in Europe. AstaCarotene AB was later purchased by the Fuji Chemical Industry (Toyama, Japan) and renamed Astareal AB. Astaxanthin dietary supplements are currently available in North America, Europe, and Asia-Pacific countries. Astaxanthin is currently produced by a number of firms, including Mera Pharmacauticals (formerly Aquasearch, Inc.), Cyanotech Corporation (Hawaii, USA), Algatechnologies (Israel), Beijing Ginkgo Group (BGG; Beijing, China), and Yunnan Alphy Biotech (Yunnan, China). Although humans have consumed algae-extracted astaxanthin for more than two decades, there have been no reports of adverse reactions to astaxanthin dietary supplements at suggested astaxanthin dosages of 2-12 mg per day.

LemnaRed® astaxanthin crystal mainly contains the free-form of the (3S, 3’S)-astaxanthin, which is identical to the astaxanthin produced by JX Nippon Oil and Energy Corporation (NDI_829); however, the natural astaxanthin-rich carotenoid extract (ARE) produced by the JX Nippon Oil and Energy Corporation contains only 60% astaxanthin, whereas LemnaRed® astaxanthin crystal contains at least 80% astaxanthin. Moreover, LemnaRed® astaxanthin softgels have been sold in Taiwan, Singapore, and Thailand since 2015. Hundreds of thousands of capsules have been sold with no reports of adverse effects.

3.1.2 History of Use of *E. coli* K-12

E. coli is a naturally occurring, gram-negative commensal inhabitant of humans and numerous animal species. Some *E. coli* strains are pathogenic; however, *E. coli* K-12 strains are non-pathogenic. Indeed, according to biological safety guidelines, they are designated as Risk Group 1 organisms, which means that they are not considered pathogenic to either humans or animals [18, 19]. *E. coli* K-12 has been used as a laboratory organism for over 50 years, and its genome was sequenced in 1997, making *E. coli* K-12 among the most widely studied microbes [20]. Resultantly, *E. coli* K-12 is one of the *E. coli* strains that is most widely used in the commercial production of food and food additives via recombinant DNA technology. *E. coli* K-12 has a well-established history of safe use, and engineered *E. coli* k-12 extracts, comprising enzymes, proteins, and other metabolites, also have an established history of safe application in food.

The first recombinant enzyme product extracted from engineered *E. coli* K-12, bovine chymosin, was approved for use in food by the FDA in 1991 [4]. Since that time, several food or food additives have been produced by and extracted from engineered *E. coli* K-12, thereby confirming the Generally Recognized as Safe (GRAS) status by the US FDA. These extracts include 1 ,3 propanediol (GRN 000302), L-leucine (GRN 000308), D-psicose 3-epimerase (GRN 000624), and 2'-O-fucosyllactose (GRN 000650). Agency response letters are included in Appendix 9. Based on the above, it can be concluded that the *E. coli* K-12 used in the production of LemnaRed® astaxanthin crystal is non-toxigenic and presents no risk the public.

In addition, astaxanthin produced by genetically modified *Escherichia coli* strain Ast12 has been considered as a food ingredient in Taiwan. A notification of "*The Draft Regulation for The Use Restrictions and Labeling Requirement of astaxanthin produced by Genetically Modified Escherichia coli strain Ast12 as a Food Ingredien*t" issued from Ministry of Health and Welfare of Taiwan to Committee on Sanitary and Phytosanitary Measures of WTO is provided in Page 53-57.

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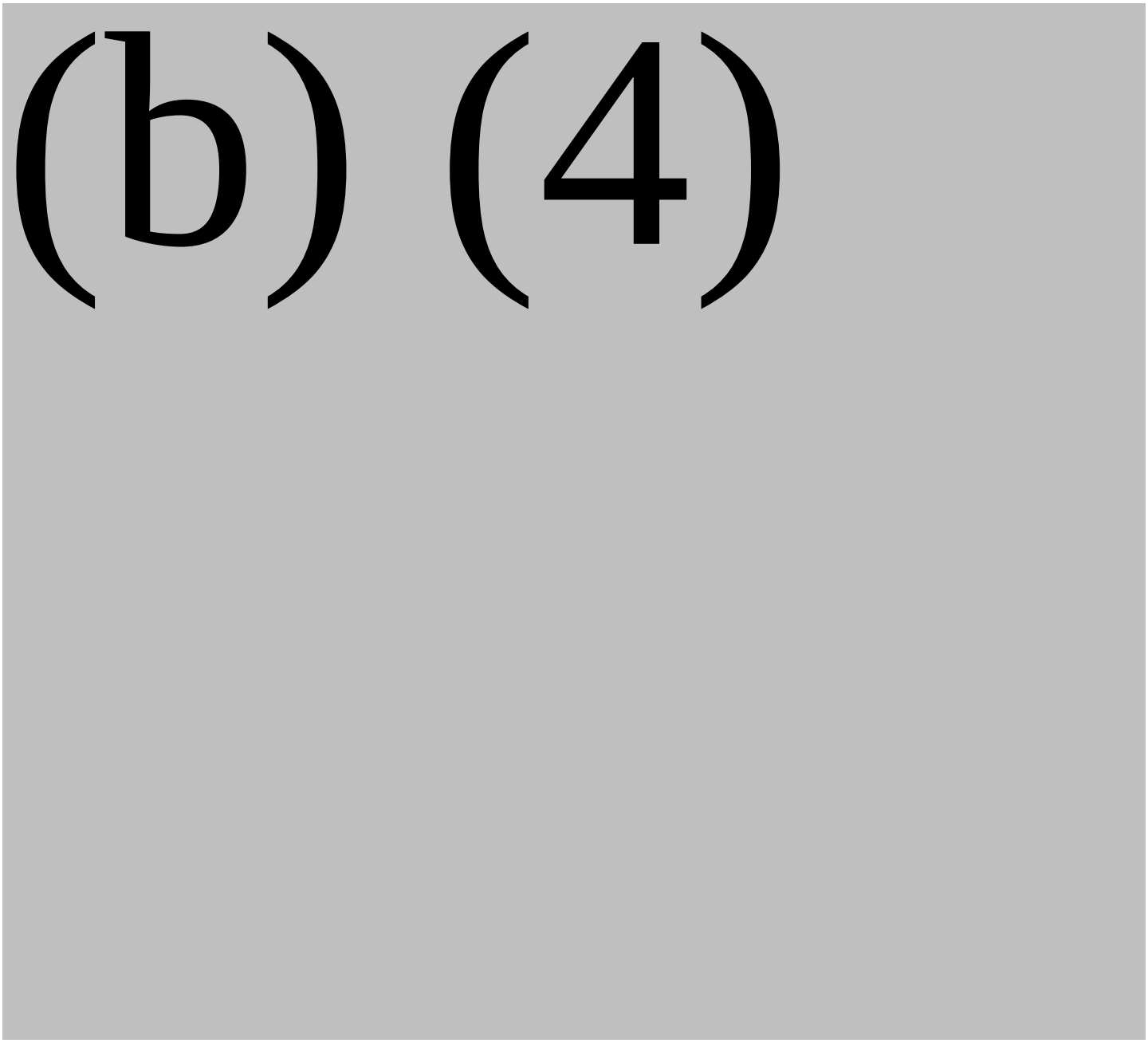
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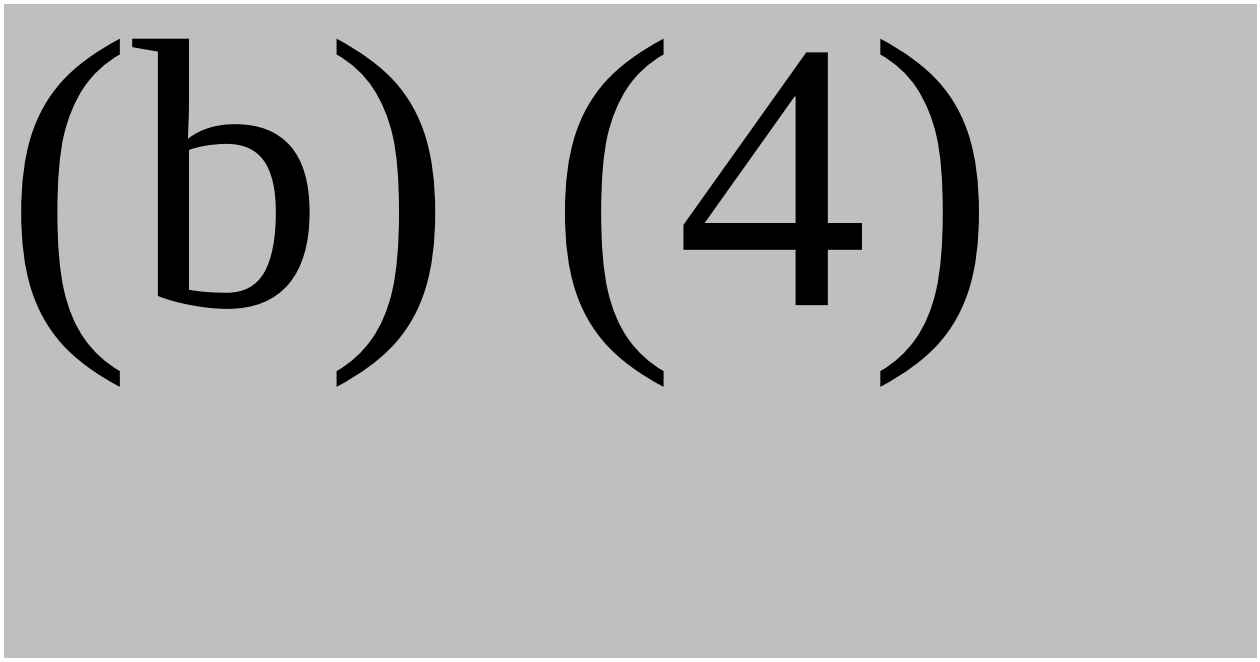
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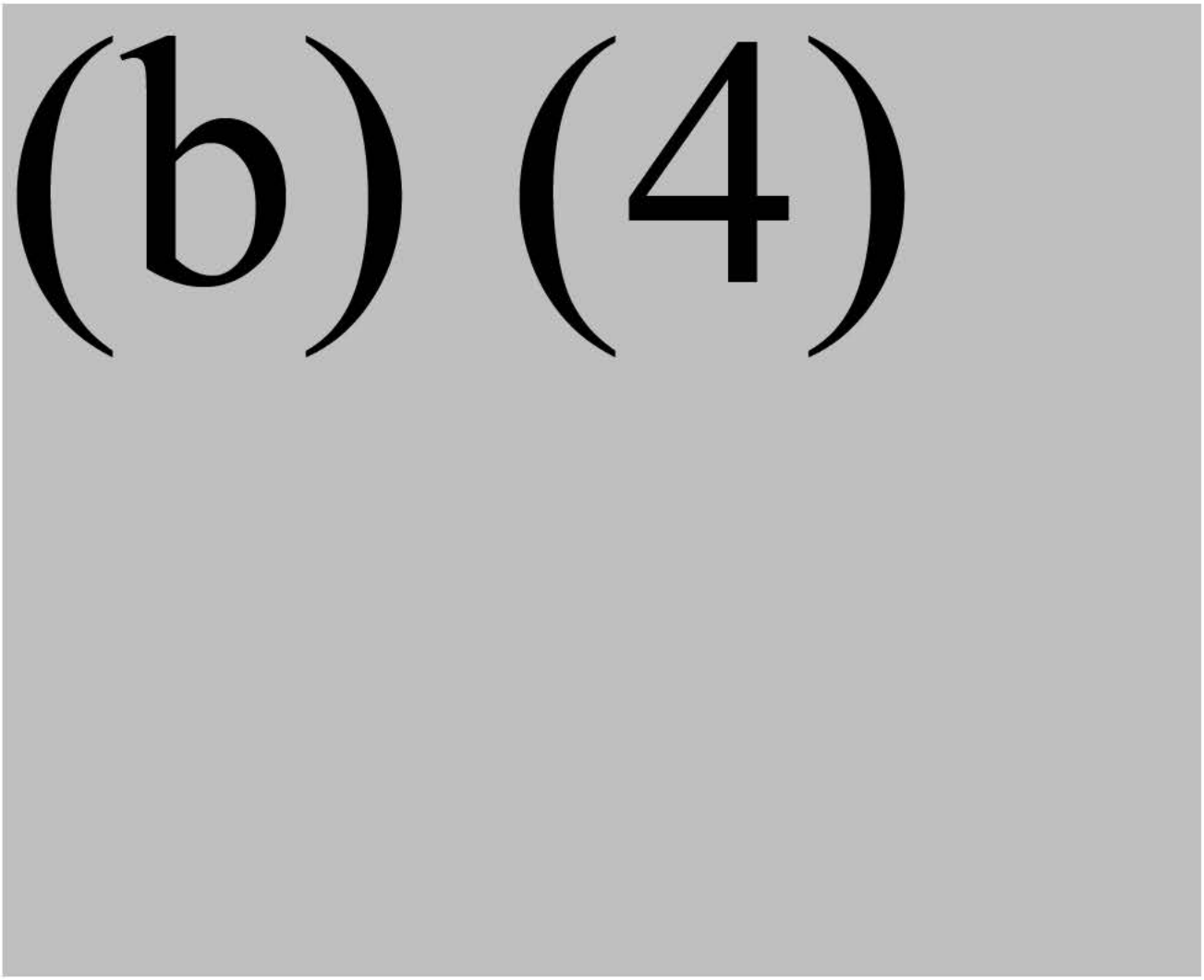
Appendix



3.1.3 Description of the relationship between the historically consumed material and the NDI or dietary supplements containing the NDI

The astaxanthin in LemnaRed® astaxanthin crystal differs in two aspects from that of the historically consumed astaxanthin found in wild salmon and algae. These two types of astaxanthin differ slightly with regard to chemical structure. The astaxanthin found in wild salmon and algae is esterified with fatty acids at one or both ends of the astaxanthin; however, the astaxanthin in LemnaRed® astaxanthin crystal is free from esterification. Esterified or not, only the free-form of the chemical can be absorbed by human body. The other difference is the purity of astaxanthin. Specifically, astaxanthin levels in wild salmon range between 1 to 58 mg/kg. In algae, the astaxanthin level is approximately 20 g/kg; however, after extraction the purity can rise to approximately 100 g/kg. In contrast, LemnaRed® astaxanthin crystal contains 800 g/kg of astaxanthin, which is much purer than that derived from algae. Therefore, LemnaRed® astaxanthin crystal is considered to be safer than the astaxanthin derived from algae.

3.1.4 Description of identity information verifying the relationship between historically consumed material and the NDI or dietary supplement containing the NDI

LemnaRed® astaxanthin crystal contains mainly free-form, (3S, 3'S)-astaxanthin, which is identical to that observed in the body of humans who have consumed wild salmon flesh or algae-derived astaxanthin supplements, such as those manufactured by Fuji Chemical Industry, Cyanotach Corporation, and Algatechnologies [21-24]. It means that the astaxanthin esters presenting in wild salmon and algae become de-esterified in the human small intestine via pancreatic esterase and lipase, thereby enabling free-form astaxanthin to be absorbed by intestinal mucosal cells. Therefore, although the astaxanthin in wild salmon and algae contains esters, it is actually free-form (3S, 3'S)-astaxanthin that is absorbed.

The chemical structure of astaxanthin in LemnaRed® crystals is identical to that produced by the JX Nippon Oil and Energy Corporation, which filed an NDI notification in 2014 (NDI_829)

3.1.5 Historical conditions of use and cumulative exposure estimates for the historically consumed material

Astaxanthin cannot be produced in the human body; therefore, it must be absorbed thorough one's diet and/or supplements. The dietary intake of astaxanthin is largely determined by consumption, which for most Americans does not exceed two-three times a week. Consuming 300 g of salmon flesh per day would result in the intake of no more than 17.4 mg of astaxanthin. Adding to this the suggested dosage of LemnaRed® astaxanthin (12 mg/day) results in a relatively low daily astaxanthin intake of approximately 30 mg/day, as outlined in our toxicity study in Section 3.2.2.

3.1.6 Adverse Events Associated With Historically Consumed Material

No adverse effects related to astaxanthin consumption have been reported.

3.1.7 Alternative Rationale for Reasonable Expectation of Safety Based on History of Use

LemnaRed® astaxanthin crystal has been marketed in Taiwan since 2015. Despite the sale of hundreds of thousands of capsules, no adverse effects have been reported.

3.2 Other Evidence of Safety

Genotoxicity studies, subchronic toxicity study in rats and prenatal developmental toxicity in rats were performed in accordance with OECD guidelines (1997) to evaluate the genotoxic potential of LemnaRed® astaxanthin crystal. The study results of subchronic toxicity study and prenatal developmental toxicity in rats were published on the journal of Regulatory Toxicology and Pharmacology[25]. In addition, other evidence for the safety of astaxanthin is also provided in this section.

3.2.1 Genotoxicity Study

3.2.1.1 Bacterial Reverse Mutation Test

Bacterial reverse mutation tests (Appendix 10) were performed in accordance with OECD guidelines (1997) to evaluate the genotoxic potential of LemnaRed® astaxanthin crystal (the subject of this NDIN). For this, five dosage levels of astaxanthin crystal (5, 2.5, 1.25, 0.625, and 0.313 mg/plate) were first tested on *Salmonella typhimurium* TA100 to determine the appropriate testing dosages for the bacterial reverse mutation test. No cytotoxic or mutagenic effects were observed. Therefore, a maximum dose of 5 mg astaxanthin crystal/plate was tested on five strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) in the presence and absence of S9 metabolic activation mixture. Under all test conditions, the number of revertant colonies was two- or three-fold less than that in the vehicle control. These results indicate that astaxanthin crystal is not mutagenic to *Salmonella typhimurium* under the test conditions of this study.

3.2.1.2 in vitro Mammalian Chromosome Aberration Test

An *In vitro* mammalian chromosome aberration test (Appendix 11) was performed in accordance with OECD (1997) guidelines to evaluate the genotoxic potential of LemnaRed® astaxanthin crystal (the subject of this NDIN). For this, six dosage levels of astaxanthin crystal (250, 125, 62.5, 31.3, 15.6, and 7.8 µg/mL) were texted for cytotoxicity by performing an MTT assay on the ovary cells of the

Chinese hamster (CHO-K1) with or without metabolic activation. Under all test conditions, cell viability was greater than 50%, which indicates that astaxanthin crystal is not cytotoxic to CHO-KI cells. Compared with negative and vehicle controls, the number of cells with chromosome aberrations was not significantly higher under any of the test conditions. In conclusion, astaxanthin crystal was shown to be non-genotoxic to CHO-K1 cells under all test conditions.

3.2.1.3 in vivo Mammalian Erythrocyte Micronucleus Test

An *in vivo* mammalian erythrocyte micronucleus test (Appendix 12) was performed in accordance with OECD (1997) guidelines to evaluate the genotoxic potential of LemnaRed® astaxanthin crystal (the subject of this NDIN). For this, CD-1®ICR mice were randomly assigned to six groups (5/group/sex), including three control groups (negative control, positive control, and vehicle control) and three astaxanthin treatment groups which received different dosage levels (500, 1000, or 2000 mg astaxanthin crystal/kg/day). In the negative control group, mice were treated with orally administered sterile water (10 mL/kg b.w.); in the positive control group, mice were treated with intraperitoneally administered cyclophosphamide (80 mg/kg b.w.); and in the vehicle control group, mice were treated with orally administered pure olive oil (10 mL/kg b.w.). All treatment and control groups were treated with only one dose. Blood samples from the tail vein were collected at 48 and 72 hours after dosing.

No significant differences in micronucleus frequency were observed between the treatment groups and negative control group. None of the astaxanthin crystal treatment groups presented a polychromatic erythrocytes percentage (PCE%) that was significantly lower than that of the vehicle control group, which indicates that astaxanthin crystal does not inhibit erythropoiesis. Moreover, no significant differences in micronucleus frequency (MN $‰_{PCE}$) were observed between any of the treatment groups and the negative control group, which indicates that astaxanthin crystal does not induce micronucleus formation. In conclusion, the astaxanthin crystal was shown to be non-genotoxic and does not affect erythropoiesis in mice under any of the test conditions.

3.2.2 Subchronic Toxicity Study in Rats

A subchronic toxicity study was also performed, wherein LemnaRed® astaxanthin crystal (extracted from engineered *E. coli*) was administered to Sprague-Dawley (SD) rats via daily oral gavage at dose levels of up to 750 mg/kg/day for a period of 90 days. The astaxanthin crystal that was investigated in this study is marketed as LemnaRed® crystal (the subject of this NDIN). In brief, animals were randomly assigned into five groups (12/group/sex), including two control groups (water for injection [WFI] and vehicle control) and three treatment groups (that received dosages of 1.2, 240.0, or 750.0 mg astaxanthin crystal/kg/day). For astaxanthin currently being marketed as a food supplement,

the suggested daily dose for a 60 kg adult is 12 mg /day (i.e., 12 / 60 kg = 0.2 mg/kg bw/day). Multiplying this value by 6 (a standard conversion factor for normalizing the dosages based on differences in the body surface area of rats and humans [26]), results in a dosage level of 1.2 mg/kg bw/day. Thus, the lowest dosage in this experiment was 1.2 mg/kg bw/day. The medium dose was 200 times higher than the lowest dose, due to the fact that a factor of 10 was used to account for variations in sensitivity within the human population, and another factor of 20 was used to predict chronic exposure in humans from a subchronic toxicity study in a single animal species. The highest concentration of the solution was treated as an upper limit. The crystals were suspended in olive oil to ensure that the solution was fluid enough to be delivered by oral gavage.

All animals survived the treatment period with no signs of toxicity. Specifically, no ophthalmological alterations were recorded prior to grouping or terminal sacrifice. Compared with the WFI control group, female rats in the mid- and high-dose groups showed significantly low body weight gain during week two, and female rats in the low-dose group showed significantly low body weight gain during week seven. However, these reductions in body weight gain fell within the range of historical control data from that lab and were therefore considered incidental; and no other statistically significant treatment-related differences were observed in terms of body weight or body weight gain among animals of either sex. In addition, food consumption was significantly lower in the vehicle control and the three astaxanthin-treatment groups rather than in the WFI control group. Nonetheless, this may have been related to the fact that olive oil accounted for a portion of the daily caloric intake. Compared to the vehicle control group, several statistical differences in food consumption were also observed between the low-dose and high-dose groups; however, no dose-dependent trend was found. Furthermore, these findings did not appear to affect body weight gain and were considered unrelated to the astaxanthin treatment.

Compared to the control groups, no treatment-related toxicologically significant changes in hematology parameters were observed. Specifically, the prothrombin time and active partial thromboplastin time of male rats in the mid-dose group were significantly lower than those in the vehicle control group but, this difference was not observed in the high-dose group. Male rats in the mid- and high-dose groups presented significantly lower prothrombin time than did those in the WFI control group. Nonetheless, red blood cell values among male rats in the mid-dose and high-dose groups were significantly higher than those in the WFI control group. Furthermore, the hematocrit and hemoglobin values of all astaxanthin-treated male rats were significantly higher than those of male rats in the WFI control group. Nonetheless, these values were still within the historical control range of the lab, as follows: hematocrit values (14.88 to 17.62 g/dL) and hemoglobin values (41.61% to 48.71). The fact that these effects were not observed in both sexes indicates that the differences we observed were not treatment-related adverse effects; i.e., they

can be considered incidental biological variations. Among female rats, the high-dose group presented statistically lower mean corpuscular hemoglobin levels, and all astaxanthin-treated female rats presented mean corpuscular hemoglobin concentrations that were statistically lower than those of the WFI control group. This effect is most likely attributable to biological differences among the animals. Furthermore, these changes were only observed among female rats, and are therefore not considered adverse effects.

Astaxanthin treatment over a period of 90 consecutive days did not have any adverse effects on the clinical chemistry parameters of rats of either sex, and all fluctuations were within the historical control data of the lab. Compared to the vehicle control group, males in the low-dose group presented significantly higher aspartate aminotransferase values and females presented significantly lower values. Conversely, the total cholesterol concentration among rats of both sexes in the high-dose group was significantly lower than that of the vehicle control group. In addition, compared to the vehicle control group, males in the low-dose and mid-dose groups presented significantly higher creatine kinase values, whereas females in the low-dose and mid-dose groups presented significantly lower values. However, blood urea nitrogen levels were significantly lower (p<0.05) among rats of both sexes in all vehicle control groups, which may have resulted from the relatively low food intake of these groups.

In conclusion, our results from a 13-week study on toxicity indicate that astaxanthin derived from engineered *E. coli* has no adverse effects on rats of either gender (see the subchronic study report in Appendix 13)). The no-observable-adverse-effect level (NOAEL) of astaxanthin derived from metabolically engineered *E. coli* was found to be 750 mg astaxanthin crystal/kg/day (equivalent to 596 mg pure astaxanthin/kg/day) in both male and female rats.

3.2.3 Prenatal Developmental Toxicity in Rats

A development toxicity study was performed, whereby astaxanthin crystal (extracted from engineered *E. coli*) was administered to pregnant Sprague-Dawley (SD) rats via daily oral gavage at dose levels of up to 750 mg/kg/day during the period of major embryonic organogenesis (G6-G15). The astaxanthin crystal that was investigated in this study is marketed as LemnaRed® crystal (the subject of this NDIN). In brief, animals were randomly mated by placing one female in a cage with one male. Vaginal smears were performed daily, and females with vaginal sperm and/or a vaginal copulation plug were removed from the cage and considered to be at Day 0 of gestation (G0).

Pregnant females were randomly assigned to five groups (N≧ 20 per group), including two control groups (water for injection [WFI] and vehicle control) and three treatment groups (that received

dosages of 1.2, 240.0, or 750.0 mg astaxanthin crystal/kg/day). The reasons for the selection of these doses are presented in Section 8.4.3.

All animals survived the treatment period, and pregnancy rates were between 89.3 and 100%. No differences in body weight gain were observed among the groups. Food consumption in the vehicle control group was statistically lower than in the WFI control group. This may be due to the fact that the vehicle control article (olive oil) accounted for a portion of the daily caloric intake. Nonetheless, all values were within normal physiological ranges and no dose-dependent trends were observed. Slight to moderate hair loss was observed in all groups: 2/28 rats in the WFI control group, 2/27 rats in the vehicle control group, 3/27 rats in the low-dose group, 2/25 rats in the mid-dose group, and 2/25 rats in the high-dose group). This is considered normal in pregnant animals and appears unrelated to the administration of astaxanthin. Tables 5 and 6 list examination results in terms of gross necropsy and reproductive parameters. In the gross examination of fetal appearance, one fetus in the vehicle-control group presented a short tail. In the fetal visceral examination, one fetus in the vehicle-control group presented a distended renal pelvis. These results indicate that astaxanthin treatment (at the dosage up to 750 mg crystal/kg/day) does not affect organogenesis and does not have toxicological potential with regard to development and reproduction (see the developmental toxicity study report in Appendix 14).

3.2.4 Discussion of Toxicity and Conclusions

Following treatment with LemnaRed® astaxanthin crystal, no adverse effects were observed in terms of body weight gain, hematology or serum chemistry values, hepatic enzyme stability, organ integrity, or fetal organogenesis. These findings indicate that LemnaRed® astaxanthin crystal provides a wide margin of safety.

3.2.5 Additional rationale for reasonable expectation of safety based on other evidence

3.2.5.1 Animal Study Conducted by Schneider, et al. (2016)

Synthetic, free-form of the (3S, 3S')-astaxanthin (with the same molecular structure as LemnaRed® astaxanthin crystal, the subject of this NDIN) was tested for developmental toxicity in an *in utero* study by Schneider et al. (2016) involving the development of New Zealand white rabbits. In that research, common variations were observed in all litters; however, when variations were examined by type and frequency, no relationship with the administration of astaxanthin was found. The authors therefore concluded that administering free-form of the (3S, 3S')-astaxanthin (in a gelatin/carbohydrate powder formulation) at a dosage level up to 400 mg/kg bodyweight/day throughout pregnancy has no harmful effects on reproduction or fetal development [27].

3.2.5.2 Animal Study Conducted by Vega, et al. (2015)

Vega et al. (2015) tested synthetic, free-form (3S, 3S')-astaxanthin with the same molecular structure as LemnaRed® astaxanthin crystal the subject of this NDIN for subchronic toxicity in Wistar rats. Those authors reported a macroscopically visible brown-blue discoloration of the gastrointestinal contents, which was deemed "secondary to the violet-brown color of the test material". However, no other significant dose-related abnormalities were observed. Therefore, results of that research indicate that ingesting synthetic, free-form of the (3S, 3S')-astaxanthin (in a gelatin/carbohydrate formulation) at a dosage level up to 700-920 mg/kg bodyweight/day does not have any adverse effects on rats [28].

3.2.5.3 Animal Study Conducted by Buesen, et al. (2015)

Buesen et al. (2015) tested all meso-forms of synthetic, free-form astaxanthin in (1) a subchronic (13-week) toxicity study and (2) a developmental toxicity study, both of which were performed on Hanlbm Wistar rats. In the subchronic study, fecal discoloration and yellow pigmentation of adipose tissue were observed. Both of those findings were shown to result from astaxanthin treatment, but were not considered a sign of toxicity. Furthermore, in the developmental toxicity study, the offspring of female rats were not adversely affected by exposure to astaxanthin (at a dosage level of 457-957 mg/kg bodyweight/day). Based on those results, the subchronic toxicity study estimated the no-observable-adverse-effect level (NOAEL) of astaxanthin to be 1033 mg/kg bodyweight/day, and the developmental toxicity study estimated the NOAEL of astaxanthin to be 830 mg/kg bodyweight/day [29].

3.2.5.4 Animal Study Conducted by Edwards, et al. (2016)

Edwards et al. (2016) tested all meso-forms of synthetic, free-form astaxanthin for genotoxic potential in a two-year mouse study involving the administration of dietary dosages of 0 (control), 0 (placebo beadlets), 40, 200, and 1000 mg astaxanthin/kg bodyweight/day. Findings of that research revealed an increase in the incidence of benign, hepatocellular adenoma at dosage levels of 200 mg/kg bodyweight/day and above; however, these effects were noted only in female animals. Histopathological examination showed that the hepatocellular adenoma in female rats was secondary to hepatotoxicity and regeneration. Furthermore, the phenomenon was considered species-specific with doubtful relevance to humans. Additionally, that research did not find any evidence of toxicity. Therefore, the authors concluded that astaxanthin is not genotoxic and does not induce mutagenic, clastogenic, or aneugenic changes [30].

3.2.5.5 Animal Study Conducted by Katsumata, et al. (2014)

Katsumata et al. (2014) tested natural, free-form (3S, 3S')-astaxanthin with the same molecular structure as LemnaRed® astaxanthin crystal (the subject of this NDIN) produced through the well-controlled fermentation of a natural bacteria, *Paracoccus carotinifaciens*. Specifically, that study tested astaxanthin for subchronic toxicity by orally administering astaxanthin-rich carotenoid extract (ARE) suspended in olive oil to Sprague-Dawley rats at daily dosages of 0 (olive oil), 250, 500, or 1000 mg/kg/day for a period of 13 weeks. Dark-red feces were observed throughout the administration period; however, no other treatment-related changes were apparent. The dark-red feces were deemed an intrinsic property of astaxanthin. The authors calculated the no observed adverse effect level (NOAEL) for ARE at no less than 1000 mg/kg bodyweight/day, which is equivalent to 596 mg of astaxanthin/kg bodyweight/day [31].

3.2.5.6 Animal Study Conducted by Tago, et al. (2014)

Tago et al. (2014) tested natural, free-form (3R, 3R')-astaxanthin derived from yeast *Phaffia rhodozyma* for its genotoxic potential and subacute toxicity. In that study, the astaxanthin did not induce reverse mutations in *Salmonella typhmurium* strains TA98 or TA100, and no chromosomal damage was observed in mouse micronucleus tests. Moreover, no evidence of subacute toxicity was observed when astaxanthin was administered to Sprague-Dawley rats in a four-week repeated oral toxicity study. Based on those results, the no observed adverse effect level (NOAEL) for astaxanthin was estimated to be greater than 1,000 mg/kg bodyweight/day [32].

3.2.5.7 Animal Study Conducted by Stewart, et al. (2008)

Stewart et al. (2008) tested a naturally esterified (3S, 3S')-astaxanthin rich biomass of microalgae *Haematococcus pluvialis* for acute and subchronic toxicity in Wistar rats. The oral LD_{50} of the biomass in rats was greater than 12g/kg body weight. In the subchronic study, some differences were reported between the test and control groups. Specifically, changes in urine parameters were observed and the high-dose treatment group showed slightly elevated alkaline phosphatase levels. Nonetheless, those differences were not considered toxicologically significant. The authors estimated the no observed adverse effect levels (NOAEL) of the astaxanthin-rich biomass for male and female rats at 14,161 and 17,076 mg/kg bodyweight/day, which are respectively equivalent to 465 and 557 mg astaxanthin/kg/day [33].

3.2.5.7 Human Study Conducted by Coombes, et al. (2016)

In a published, randomized, placebo-controlled, double-blind clinical trial by Coombes et al. (2016), 61 patients that had undergone a renal transplant orally received either 12 mg astaxanthin/day or

an identical placebo over a period of one year. The astaxanthin (provided by the Cyanotech Corporation) was derived from *Haematococcus pluvialis*. In that study, astaxanthin had no effect on arterial stiffness, oxidative stress, or inflammation in renal transplant recipients. Furthermore, no intervention-related adverse events were noted, indicating that the consumption of 12 mg of astaxanthin/day could be considered safe [34].

3.2.5.8 Human Study conducted by Kim, et al. (2011)

Kim et al. (2011) examined the antioxidant potential of *Haematococcus* astaxanthin on oxidative stress in smokers, whereby 39 heavy smokers (≧20 cigarettes/day) and 39 non-smokers were randomly divided into three dosage groups. Each group received astaxanthin at dose of 5, 20, or 40 mg/day for a period of three weeks. Astaxanthin levels in plasma and oxidative stress biomarkers, such as malondialdehyde, isopeostane, superoxide dismutase and total antioxidant capacity, were measured at the beginning of the treatment period and also during weekly treatments. Over the three-week period, the superoxide dismutase level and total antioxidant capacity increased in all astaxanthin treatment groups, whereas the plasma malondialdehyde level decreased. Moreover, isoprostane levels showed a particularly pronounced dose-dependent decrease following astaxanthin intake. These results suggest that (1) a daily dose of 40 mg astaxanthin could be safe for humans and (2) astaxanthin supplements may prevent oxidative damage in smokers by suppressing lipid peroxidation and stimulating the antioxidant system [35].

3.2.5.9 Human Study Conducted by Nakagawa, et al. (2011)

Nakagawa et al. (2011) investigated the efficacy of astaxanthin supplements (containing 6 or 12 mg of astaxanthin oil derived from *Haematococcus pluvialis)* in a published, randomized, placebo-controlled, double-blind clinical trial. In that research, a total of 30 healthy subjects between 50 and 69 years of age were randomly divided into three groups to undergo astaxanthin treatment at three dosage levels (0, 6, or 12 mg). After 12 weeks, the treatment groups presented erythrocyte astaxanthin concentrations that were higher than those in the placebo group and erythrocyte phospholipid hydroperoxide (PLOOH) concentrations that were lower than those in the placebo group. The authors concluded that the astaxanthin had been incorporated into erythrocytes. The authors had previously confirmed abnormally high levels of PLOOH in the erythrocytes of dementia patients. Thus, the observed decrease in erythrocyte PLOOH levels indicates that the administration of astaxanthin may help to prevent dementia. Finally, the lack of adverse effects during the trial led the authors to conclude that a daily intake of 12 mg astaxanthin is safe for humans [36].

3.2.5.10 Human Study Conducted by Yoshida, et al. (2010)

In a published, randomized, placebo-controlled, double-blind clinical trial by Yoshida et al. (2010), 61 healthy, non-obese subjects between 25 and 60 years of age received astaxanthin supplements (derived from *Haematococcus pluvialis*) at dosage levels of 0, 6, 12, or 18 mg/day over a period of 12 weeks. Those authors reported that astaxanthin supplements significantly decreased triglyceride levels and increased HDL-cholesterol and adiponectin in humans. The authors therefore concluded that astaxanthin could be used to treat impaired lipid metabolism and prevent atherosclerosis. A lack of adverse effects during the trial also led the authors to conclude that a daily intake of 12 mg astaxanthin is safe [37].

3.2.5.11 Human Study Conducted by Satoh, et al. (2009)

This report by Satoh et al. (2009) included two open-label clinical studies. One study evaluated the efficacy of astaxanthin extracted from *Haematococcus pluvialis* in the treatment of age-related forgetfulness, and the other study assessed clinical toxicity. In the efficacy study, 10 otherwise healthy male subjects who complained of age-related forgetfulness ingested 12 mg of astaxanthin once daily over a period of 12 weeks. CogHealth and P300 results from that research indicated that, in older individuals, astaxanthin can improve higher brain functions with subsequent effects on behavior. In the toxicity study, 127 subjects between 20 and 60 years of age were divided into three groups. Each group was respectively administrated 4, 8, or 20 mg of astaxanthin once per day over a period of four weeks. No signs of abnormality were observed in the toxicity study at any dosage level. Finally, Satoh et al. (2009) determined that an intake dosage of 12 mg astaxanthin per day could be regarded as safe [24].

3.2.5.12 Human Study Conducted by Iwabayashi, et al. (2009)

In an open-label noncontrolled study by Iwabayashi et al. (2009), 35 healthy postmenopausal women received a daily dose of 12 mg astaxanthin (extracted from *Haematococcus pluvialis*) over a period of eight weeks. Researchers administered the anti-aging QOL Common Questionnaire and examined somatometry, hematology, oxidative stress, and vascular function before treatment began and after subjects had received astaxanthin for four and eight weeks. Results indicated that astaxanthin may enhance antioxidant capacity, reduce vascular resistance in the lower limbs, decrease blood pressure, and improve physical symptoms in woman with high oxidative stress. The results also suggested that a daily dose of 12 mg astaxanthin could be regarded as safe for healthy postmenopausal women [38].

3.2.5.13 Human Study Conducted by Andersen, et al. (2007)

Andersen et al. (2007) examined 44 patients (6 males and 38 females with an age range between 20 and 70 years old) suffering from functional dyspepsia. Based on a urea breath test, histology, or cultures, 29 of these patients were identified as positive for *Helicobacter pylori* and 15 of these patients were identified as negative. Patients were randomly assigned to two groups and, for a period of four weeks, were blindly treated with (1) five placebo capsules twice daily or (2) five capsules, each of which contained 4 mg *Haematococcus pluvialis*-derived astaxanthin, twice daily. Following treatment, a significant decrease in gastric inflammation was observed in *H. pylori*-positive patients in both groups; however, no changes in the density of *H. pylori* or in any of the interleukins were observed. No adverse effects of astaxanthin treatment at a daily dosage of 40 mg were noted, indicating that our suggested dosage of 12 mg of astaxanthin per day could be regarded as safe [39].

3.2.5.14 Human Study Conducted by Comhaire, et al. (2005)

In a randomized, placebo-controlled, double-blind clinical trial by Comhaire et al. (2005), 30 men who had suffered from infertility for over 12 months received conventional treatment (in accordance with guidelines proposed by the World Health Organization; WHO) in conjunction with either *Haematococcus pluvialis*-derived astaxanthin (16 mg/day) or a placebo for a period of 3 months. In that study, the effects of treatment on semen parameters, reactive oxygen species (ROS), a Zona-free hamster oocyte test, serum hormones, and spontaneous or intrauterine insemination (IUI)-induced pregnancies were evaluated. Results demonstrated that astaxanthin can (1) decrease ROS and the hormone Inhibin B and also (2) increase the linear velocity of sperm. Results of that study also indicate that our suggested dosage of 12 mg of astaxanthin per day could be regarded as safe [40].

3.2.5.15 Human Study Conducted by Iwamoto, et al. (2000)

Iwamoto et al. (2000) conducted *in vitro* and *ex vivo* studies to determine the effects of astaxanthin on LDL oxidation. (The astaxanthin used in that research was extracted from krill, and most of it was in the esterified form.) In the *ex vivo* study, 24 volunteers were treated with a daily dosage of up to 21.6 mg astaxanthin over a period of 14 days. At the end of the study period, the authors concluded that astaxanthin inhibits LDL oxidation and may help prevent atherosclerosis. Their findings also indicate that our suggested dosage of 12 mg of astaxanthin per day (far below the highest dosage of 21.6 mg astaxanthin used in that study) could be regarded as safe [41].

3.2.5.16 Conclusion of Additional Rationale for Reasonable Expectation of Safety Based on Other Evidence

Astaxanthin can be produced at the industrial scale using microalgae, yeast, bacteria or through chemically synthesis. The astaxanthin from each if these sources have been studied with regard to toxicity, and no adverse effects have been reported thus far. Moreover, clinical studies on the effects of astaxanthin have been conducted on subjects of various nationalities and ages. Despite the fact that some of the studies reported no beneficial effects, none of the studies have reported adverse effects. Thus, the consumption of astaxanthin at a recommended daily dosage of 12 mg/day should be regarded as safe.

4. Basis For Concluding That the New Dietary Ingredient Could Reasonably Be Regarded as Safe For Use in a Dietary Supplement (Required)

4.1 Determination of the No-Observed-Adverse-Effect-Level (NOAEL) or Lowest-Observed Adverse Effect Level (LOAEL)

Based on a 90-day subchronic toxicity study of LemnaRed® astaxanthin crystal (Appendix 13), a NOAEL of 750 mg/kg bw/day in rats is purposed.

4.2 Determination of Safety Factors

Based on recommendations in "New Dietary Ingredient Notifications and Related Issues: Guidance for Industry", we selected the safety factors Uf_{intra}, Uf_{inter}, and Uf_{extra}, and multiplied them to create a combined safety factor, as follows: $Uf_{intra} \times Uf_{inter} \times Uf_{extra.}$

where Uf_{intra} is a factor of 10 that accounts for variations in sensitivity within the human population, and Uf_{inter} is a factor of 10 that accounts for interspecies variations between animals and humans, and Uf_{extrap} is a factor of 20 that predicts chronic exposure in humans from a subchronic toxicity study in a single animal species. Thus, the combined safety factor is $10 \times 10 \times 20 = 2000$.

4.3 Determination of the Acceptable Daily Intake (ADI)

Based on recommendations in "*New Dietary Ingredient Notifications and Related Issues: Guidance for Industry*", we calculated the acceptable daily intake (ADI) of LemnaRed® using the followed formula: ADI= NOAEL/combined safety factor.

Thus, the ADI of LemnaRed® crystal is 750/2000= 0.375 mg/kg/day.

4.4 Determination of Estimated Daily Intake (EDI) and the EDI/ADI Ratio

The suggested daily dose of LemnaRed® crystal is not more than 15 mg/day, which is equivalent to 12 mg astaxanthin/day. Based on recommendations in "*New Dietary Ingredient Notifications and Related Issues: Guidance for Industry*", we estimated the daily intake (EDI) at 15 mg/person/70 kg average adult= 0.21 mg/kg.

4.5 Determination of Margin of Safety

Based on recommendations in "*New Dietary Ingredient Notifications and Related Issues: Guidance for Industry*", we obtained an EDI/ADI ratio of 0.21/0.375= 0.56, which provides a significant margin of safety.

4.6 Safety Narrative and Conclusion

The safety of LemnaRed® astaxanthin crystal is supported by the following:

1. Genotoxicity study of LemnaRed® astaxanthin crystal (section 3.2.1)
2. Subchronic toxicity study of LemnaRed® astaxanthin crystal in rats (section 3.2.2)
3. Developmental toxicity study of LemnaRed® astaxanthin crystal in rats (section 3.2.3)

Based on a critical evaluation of the data in the above-mentioned studies, we conclude that the LemnaRed® astaxanthin crystal with the specifications cited in Section 1.3.4, when used as a dietary supplement at a maximum daily dose of 12 mg of pure astaxanthin/day, can reasonably be regarded as safe.

4.7 Alternative Basis for Reasonable Expectation of Safety

The following reports also support the safety of LemnaRed® astaxanthin crystals:

1. Developmental toxicity study of free-form, (3S, 3'S)-astaxanthin in New Zealand white rabbits (section 3.2.5.1)
2. Subchronic toxicity study of free-form, (3S, 3'S)-astaxanthin in rats (section 3.2.5.2)
3. Subchronic toxicity study of fermentation-derived, free-form, (3S, 3'S)-astaxanthin in rats (section 3.2.5.5)
4. Previous NDI notification No. 829, titled "Astaxanthin-rich Carotenoid Extract [ARE] from JX Nippon Oil and Energy Corporation". The ARE in that notification is mainly free-form, 3S, 3'S-astaxanthin and was produced through the same fermentation process as that used by LemnaRed® astaxanthin crystal.
5. Report by Osterlie, M. et al. (2000) which revealed that free-form astaxanthin does not bioaccumulate in the body. Specifically, results of that report indicated that the maximum levels of astaxanthin (1.3 ± 0.1 mg/L) were reached 6.7±1.2 hours following the ingestion of 100 mg astaxanthin. The plasma astaxanthin elimination half-life was calculated at 21 ± 11 hours.

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