SCG
Spherix Consulting Group

September 21, 2022

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
CPK-2 Building, Room 2092
5001 Campus Drive, HFS-225
College Park, MD 20740

Dear GRAS Filing Team:

Enclosed please find a CD containing "Generally Recognized As Safe (GRAS) Notification for the Use of ARA-rich Oil As An Ingredient in Exempt and Non-Exempt Infant Formula", Form 3667, and all corresponding references. The data and information that serve as the basis for this GRAS notification is available for review and copying at reasonable times at the office of Claire Kruger, PhD, DABT, Managing Partner, Spherix Consulting Group, Inc., 751 Rockville Pike, Unit 30-B, Rockville, MD 20852, Telephone: 301-775-9476; Email: ckruger@spherixgroup.com, or will be sent to FDA upon request.

It is our opinion that the enclosed GRAS Notice constitutes a new notification because Hubei Fuxing Biotechnology Co., Ltd utilizes a novel strain of Mortierella alpina, M. alpina AF, in the production of ARA-rich oil.

We thank you for taking the time to review this GRAS notification. Should you have additional questions, please let us know.

Sincerely,

Claire L. Kruger, PhD, DABT, CFS
Managing Partner

Enclosure:

CD containing Form 3667, cover letter, and GRAS Notification for the Use of ARA-rich Oil As An Ingredient in Exempt and Non-Exempt Infant Formula, and all references

GENERALLY RECOGNIZED AS SAFE (GRAS) NOTIFICATION FOR THE USE OF ARA-RICH OIL AS AN INGREDIENT IN EXEMPT AND NON-EXEMPT INFANT FORMULA

Prepared for:

Hubei Fuxing Biotechnology Co., Ltd.
No. 18 Fuxing Ave
Chenhu Town, Hanchuan City 431608
Hubei Province, China

Prepared by:

Spherix Consulting Group, Inc.
751 Rockville Pike, Unit 30-B
Rockville, MD 20852

September 7, 2022

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AOCS: American Oil Chemist's Society

ARA: Arachidonic acid

CCP: Critical control point

CFR: Code of Federal Regulations

CFU: Colony forming units

DHA: Docosahexaenoic acid

EDI: Estimated daily intake

EU: European Union

EFSA: European Food Safety Authority

FCC: Food Chemicals Codex

FDA: Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FOIA: Freedom OF Information Act

FSANZ: Food Standards of Australia and New Zealand

GMP: Good manufacturing practice

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

ISO: International Organization for Standardization

LCPUFA: Long-chain polyunsaturated fatty acids

LOQ: Limit of quantitation

meq: Milliequivalents

NMKL: Nordic Committee on Food Analysis

NOAEL: No observed adverse effect level

PAH: Polyaromatic hydrocarbons

PCBs: Polychlorinated biphenyls

PUFA: Polyunsaturated fatty acid (PUFA

PPARγ: Peroxisome proliferator-activated receptor γ

I. SIGNED STATEMENT OF THE CONCLUSION OF GENERALLY RECOGNIZED AS SAFE (GRAS) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260

A. SUBMISSION OF GRAS NOTICE

Hubei Fuxing Biotechnology Co., Ltd. is hereby submitting a GRAS Notice in accordance with subpart E of part 170 of Title 21 of the United States Code of Federal Regulations.

B. NAME AND ADDRESS OF THE SPONSOR

Hubei Fuxing Biotechnology Co., Ltd.
No. 18 Fuxing Ave,
Chenhu Town, Hanchuan City 431608, Hubei Province,
China

C. COMMON OR USUAL NAME

Arachidonic acid (ARA)-rich oil, or ARA-rich oil

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

This notification does not contain any trade secrets or confidential information.

E. INTENDED USE

ARA-rich oil is intended for use as an ingredient in exempt infant formula that will be consumed by preterm infants, as well as non-exempt infant formula for term infants. ARA-rich oil will be consumed in combination with a safe and suitable source of DHA, consistent with the generally accepted and current guidelines for safe infant feeding practices.

F. BASIS FOR GRAS DETERMINATION

This GRAS Notice for the use of ARA-rich oil as an ingredient in infant formula is based upon scientific procedures as described under 21 CFR §170.30(b). The intake of ARA-rich oil from the intended uses specified above has been determined to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), Section 201(s). The GRAS Notice is made on the basis of generally available and accepted information evaluated by independent experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food.

Hubei Fuxing Biotechnology Co., Ltd is proposing to market ARA-rich oil, produced by Hubei Fuxing Biotechnology Co., Ltd, China, as a source of ARA-rich oil used in the manufacture of infant formula. The end-use infant formulas are exempt pre-term infant formula and non-exempt term infant formula. Consistent with other GRAS sources of ARA-rich oil GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001), this ingredient is produced by the fungus *Mortierella alpina* and specifications stipulate a minimum of 40% arachidonic acid in the oil.

The following safety evaluation considers the composition, intake, nutritional, microbiological, and toxicological properties of Hubei Fuxing's ARA-rich oil based on publicly available data from essentially equivalent ARA-rich oils as determined GRAS in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001), each of which received "no questions" letters from the United States Food and Drug Administration (FDA). The proposed use of Hubei Fuxing's ARA-rich oil as an ingredient in non-exempt term infant formula and exempt pre-term infant formula has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based upon the following:

- The compositional data and product specifications are demonstrative of carefully controlled production and refining processes.

- The morphology, biochemistry, and physiology of *M. alpina* are well-documented and it is not pathogenic or toxic. The *M. alpina* strain used in the production of ARA-rich oil is not genetically modified.

- The FDA has issued 'no question' letters for six GRAS notices for ARA-rich oils derived from *M. alpina* for infant formula: GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001). A comparison of the specifications among the ARA-rich oil that is the subject of this notification and those in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001) demonstrate that the product specifications for Hubei Fuxing's ARA-rich oil are comparable to the product specifications for the ARA-rich oil generated from *M. alpina* as described in those GRNs, with some parameters being more stringently controlled, including acid value, anisidine value, mercury, and moisture. Specifications for ARA-rich oils determined GRAS in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001) show that they are similar in composition to the ARA rich oil produced by Hubei Fuxing. Accordingly, the information described in these ARA-rich oil GRNs is relevant as supportive data for the subject of this Notice.

- The intended use for ARA-rich oil is as a source of ARA in exempt and non-exempt infant formulas. The functional importance of long-chain polyunsaturated fatty acids (LCPUFA) in pregnancy, lactation, and infancy has been the subject of numerous clinical evaluations, particularly as they relate to the intake of the n-6 and n-3 LCPUFAs, ARA (C20:4n-6) and docosahexaenoic acid (DHA; C22:6n-3). Human studies indicate that infants may not synthesize sufficient amounts of ARA and DHA *de novo* from their respective precursors to cover the high demand during this period of rapid accretion for critical normal growth and development. Because breastfeeding and human milk are the normative standards for infant feeding and nutrition (American Academy of Pediatrics Policy, 2012), infant formula must be capable of supporting the nutritional needs for the growth and development of pre-term and term infants when infant formula is chosen as a surrogate for human milk. Based on current knowledge regarding the importance of LCPUFA in infant nutrition, their presence in human milk, and the guidelines and recommendations established by the European Academy of Paediatrics, World Association of Perinatal Medicine, the Early Nutrition Academy, and the Child Health Foundation, LCPUFAs should comprise 0.3 to 0.5% by weight of the total dietary fat of infant formula, with the minimum amount of ARA being equivalent to the DHA content.

- The intended use of ARA-rich oil is to provide a source of ARA in infant formula at a concentration consistent with that of human milk. The proposed use of ARA-rich oil is intended to provide ARA at 0.75% and 0.40% by weight of fatty acids in term and pre-term infant formulas, respectively. This is within the range of ARA found in human milk. The intended use of ARA-rich oil to deliver this concentration of ARA corresponds to 1.875% by weight of total fat for non-exempt term infant formula and 1.0% by weight of total fat for exempt pre-term infant formula. This intended use level is consistent with the levels of use currently recommended by the European Academy of Paediatrics and the Child Health Foundation.

- An estimate of exposure to ARA from its addition to infant formula at target ARA levels of 0.75 g and 0.40 g per 100 g total fat for term and pre-term infant formulas may be calculated as follows: assuming human infants consume 100 kcal/kg body weight/day (term infants) to 120 kcal/kg body weight/day (pre-term infants), of which fat comprises about 50% of the energy, an infant will consume 5.6 g (term infants) to 6.7 g (pre-term infants) of fat/kg body weight/day (1 g fat = 9 kcal). These amounts correspond to intakes of ARA of 42 mg and 27 mg ARA/kg body weight/day (or 105 and 67 mg of ARA-rich oil/kg body weight/day) for term infants and pre-term infants, respectively.

- The source organism, manufacturing process, product specifications, and intended uses of Hubei Fuxing's ARA-rich oil are essentially equivalent to ARA-rich oil cited in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001); therefore, publicly available animal and human safety and tolerance studies of this ARA-rich oil support the safety of Hubei Fuxing's ARA-rich oil.
    - The safety of ARA-rich oils as ingredients in infant formula has been reviewed by numerous regulatory bodies worldwide. In these jurisdictions, the conclusions reached were that ARA-rich oils derived from *M. alpina*, meeting appropriate food grade specifications, provide a safe ARA source for supplementation of infant formula. These decisions have led to its availability for this use in at least 50 countries worldwide.
- Numerous in vitro and in vivo safety studies have been conducted over a period of more than two decades on ARA-rich oils derived from *M. alpina*. ARA-rich oil is not genotoxic as assessed by multiple genotoxicity assay. The toxicology studies cited in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001) include four subchronic toxicology studies with an *in utero* exposure which establish a range of no-observed adverse event levels (NOAELs) of 1.5-5% ARA-rich oil in the diet, or 374-3170 mg/kg/day (Hempenius, Lina, and Haggitt 2000; Casterton et al. 2009; Gao et al. 2014; Lina et al. 2006). Additional toxicology studies showed a lack of adverse effects on developmental or reproductive parameters. Tolerance and safety in a neonatal piglet model determined that dietary ARA concentration of up to 96 mg ARA/100 kcal was safe and well tolerated.
    - A corroborative, unpublished bacterial reverse mutation assay of Hubei Fuxing's ARA-rich oil was negative.
- There were no test article-related adverse effects reported in clinical studies of infant formula containing fungal-derived ARA-rich oils in pre-term infants when used at levels up to 0.91% total fatty acid content.
- Clinical studies, detailed in GRN 963 (2021) and GRN 326 (2010) using 0.64-0.72% of total fatty acids as ARA also confirmed the safety of infant formula containing fungal-derived ARA-rich oil in term and preterm infants.

Taken together, the available data from studies conducted on ARA-rich oils from *M. alpina* establish a strong body of evidence for the safety of ARA-rich oil as a source of ARA for supplementation of infant formula.

The GRAS status of ARA-rich oil (compliant with the established food grade specifications), under the intended conditions of use proposed by Hubei Fuxing, has been determined through the deliberations of Roger Clemens, DrPH, CNS, CFS, FACN, FIFT, A. Wallace Hayes, PhD, DABT, FATS, ERT, CNS, FACN, and Thomas Sox PhD, JD. These individuals are qualified by scientific training and experience to evaluate the safety of food and food ingredients. These experts have carefully reviewed and evaluated the publicly available information summarized in this document, including the safety of ARA-rich oil and the potential human exposure to ARA-rich oil resulting from its intended use as an ingredient in infant formula, and have concluded:

> *There is no evidence in the available information on ARA-rich oil that demonstrates, or suggests reasonable grounds to suspect, a hazard to the public when ARA-rich oil from M. alpina as produced by Hubei Fuxing Biotechnology Co., Ltd. is used at levels that might reasonably be expected from the proposed applications. ARA-rich oil from M. alpina as produced by Hubei Fuxing Biotechnology Co., Ltd. is GRAS for use as an ingredient in the manufacture of infant formula.*

ARA-rich oil is thus safe and GRAS at the proposed levels of ingestion. It is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

G. PREMARKET APPROVAL

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of intended use.

H. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS Notice will be available for review and copying at reasonable times at the office of Claire L. Kruger, PhD, DABT, Managing Partner, Spherix Consulting Group, Inc., at 751 Rockville Pike, Unit 30-B, Rockville, MD 20852. Telephone: 301-775-9476; Email: ckruger@spherixgroup.com, or be sent to FDA upon request.

I. FREEDOM OF INFORMATION ACT (FOIA)

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA.

J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION

To the best of our knowledge, the information contained in this GRAS notification is complete, representative, and balanced. It contains both favorable and unfavorable information, known to Hubei Fuxing Biotechnology Co., Ltd. and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

Signature of Authorized Representative of
Hubei Fuxing Biotechnology Co., Ltd.

2022.9.20

Date

II. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

A. COMMON OR USUAL NAME

Arachidonic acid (ARA)-rich oil, or ARA-rich oil

B. TRADE NAME

Arachidonic Acid Oil

C. DESCRIPTION OF ARA-RICH OIL

Arachidonic acid (ARA, 20:4 n-6)-rich oil is a source of ARA in infant formula, produced by the fungus *Mortierella alpina*, and consists of at least 40% ARA in addition to other long chain saturated and unsaturated fatty acids.

1. Background on Arachidonic Acid

Arachidonic acid is an n-6 polyunsaturated fatty acid (PUFA) found in the phospholipids of the cell membrane and is particularly abundant in the brain, muscles, and liver. Arachidonic acid is a precursor of eicosanoids, important signaling molecules that include prostaglandins, thromboxanes, and leukotrienes. Virtually all cellular ARA is esterified in membrane phospholipids where its localization is tightly regulated through multiple interconnected pathways (Hadley et al. 2016).

Arachidonic acid is not an essential fatty acid, meaning that humans can synthesize it from other PUFAs, such as linoleic acid. Long chain PUFAs are essential for the normal development of infants and children. Arachidonic acid is a key nutrient in human breast milk, as both term and pre-term infants have limited ability to convert the precursor PUFAs to ARA, due to reduced concentrations and activity of desaturase enzymes (Hadley et al. 2016; Martin et al. 2011). The supplementation of infant formula with ARA at levels consistent with those in human milk is important because n-6 and n-3 fatty acids present in human milk have critical roles in membrane structure and as precursors of potent and highly reactive eicosanoids (Hadley et al. 2016). Arachidonic acid begins to accumulate in neuronal tissue in the developing fetus during the third trimester, and gradually increases until it plateaus around age 4. Arachidonic acid is one of the most abundant fatty acids in the brain and is present in similar quantities to another PUFA, docosahexaenoic acid (DHA).

2. Source and Strain Identity of the Production Organism

Mortierella alpina is in the Zygomycetes Class in the Zygomycota Order. All Zygomycetes share two properties that readily distinguish them from the remaining classes of fungi. First, their asexual spores are endogenously formed, and second, their mycelium shows no cross walls except in regions where a specialized cell is formed from a hyphal tip (non-septate mycelium). The Zygomycetes include a group of soil inhabitants known as terrestrial Zygomycetes. The organism is easily isolated from the soil and has been identified in soils from diverse geographies (Deacon, 2006). Like many fungi, *M. alpina* is associated with common root crops and therefore, is in the direct food chain of many mammals.

Mortierella species have been widely studied in isolated laboratory culture and their morphology, biochemistry, and physiology are well documented. *Mortierella alpina* has been described in Japanese publications and patents as a potential source of ARA and, consequently, it has been the subject of many intensive laboratory investigations (Totani and Oba 1987; Shinmen et al. 1989; Bajpai, Bajpai, and Ward 1991; Lindberg and Molin 1993), none of which have reported *M. alpina*-associated pathogenicity or toxigenicity to humans or animals (Domsch et al., 1980; Scholer et al., 1983).

ARA-rich oil that is the subject of this GRAS Notice is manufactured from *M. alpina* strain AF, endogenously found in soil, isolated by Huazhong University of Science and Technology, and used by Hubei Fuxing. The strain was deposited on March 10, 2003, at the Type Culture Collection Committee of the Chinese Academy of Sciences (Deposit No.: CGMCC No. 0903). *Mortierella alpina* strain AF has been cultured at Hubei Fuxing Biotechnology Co. since 2003.

Mortierella alpina AF was verified to be *M. alpina* through analysis of the *18S* and *26S* rRNA gene sequences. The sample submitted for analysis was from a slant culture used to maintain the strain before being used in the production process. The *18S* and *26S* genes were sequenced, then those sequences were submitted to BLAST (basic local alignment sequencing tool) analysis to verify that the genes are identical to the *18S* and *26S* sequences for *M. alpina*.

The *18S* rRNA sequence of *M. alpina* AF was 100% identical to other *M. alpina* strain *18S* rRNA sequences (Table 1). The gene hits with 100% sequence identity included three hits from the *18S* gene in *M. alpina* (bolded text).

Table 1. Top BLAST Hits for *18S* rRNA Sequencing Results from Hubei Fuxing's *M. alpina* AF

NCBI Accession Number	Description*	Max Score	Total Score	Query Cover	E value	Identity
AB476409.1[1]	***Mortierella alpina* gene for *18S* rRNA, partial sequence**	1397	1397	100%	0.0	100%
AJ271630.1[2]	***Mortierella alpina 18S* rRNA gene (partial), *5.8S* rRNA gene, *26S* rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), strain CBS 224.37**	1397	1397	100%	0.0	100%
AJ271629.1[2]	***Mortierella alpina 18S* rRNA gene (partial), *5.8S* rRNA gene, *26S* rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), strain CBS 528.72**	1397	1397	100%	0.0	100%
KJ890360.1[3]	*Mortierellales sp. AGED 18S* ribosomal RNA gene, partial sequence	1397	1397	100%	0.0	100%
AB534492.1[4]	Uncultured fungus gene for *18S* rRNA, partial sequence, clone: I_3_76	1397	1397	100%	0.0	100%
AB521052.1[5]	*Mortierella sp. CO-21* gene for *18S* ribosomal RNA, partial sequence	1397	1397	100%	0.0	100%

*Bolded text are hits from *M. alpina*
NCBI: National Center for Biotechnology Information
[1]Sekiguchi H. et al., 2009, unpublished, direct submission to NCBI
[2]Mackenzie et al. 2000
[3]Tan L. et al., 2014, unpublished, direct submission to NCBI
[4]Takada Hoshino and Morimoto 2010
[5]Tagawa et al. 2010

The *26S* rRNA sequence from *M. alpina* AF was 99% identical to *26S* rRNA *M. alpina* genes (Table 2, bolded text), as well as *M. polygonia* and *M. globalpina*, which are closely related to *M. alpina* (Wagner et al. 2013).

Table 2. Top BLAST Hits for *26S* rRNA Sequencing Results from Hubei Fuxing's *M. alpina* AF

NCBI Accession Number	Description	Max Score	Total Score	Query Cover	E value	Identity
LC125559.1[1]	***Mortierella alpina* gene for *26S* rRNA, partial sequence, strain: AB14-3**	1327	1327	100%	0.0	99%
LC125558.1[1]	***Mortierella alpina* gene for *26S* rRNA, partial sequence, strain: AB14-1**	1323	1323	100%	0.0	99%
LC125544.1[1]	***Mortierella alpina* gene for *26S* rRNA, partial sequence, strain: AB7-2**	1317	1317	100%	0.0	99%
LC125553.1[1]	***Mortierella alpina* gene for *26S* rRNA, partial sequence, strain: AB11-6**	1314	1314	100%	0.0	99%
LC125551.1[1]	***Mortierella alpina* gene for *26S* rRNA, partial sequence, strain: AB11-3**	1314	1314	100%	0.0	99%
LC125542.1[1]	***Mortierella alpina* gene for *26S* rRNA, partial sequence, strain: AB6-11**	1314	1314	100%	0.0	99%
JN940866.1[2]	***Mortierella alpina* strain CBS 210.32 *28S* ribosomal RNA (LSU) gene, partial sequence**	1284	1284	100%	0.0	99%
LC125576.1[1]	*Mortierella polygonia* gene for *26S* rRNA, partial sequence, strain: AB16-17	1280	1280	100%	0.0	99%
LC125547.1[1]	*Mortierella polygonia* gene for *26S* rRNA, partial sequence, strain: AB7-7	1280	1280	100%	0.0	99%
LC125543.1[1]	*Mortierella polygonia* gene for *26S* rRNA, partial sequence, strain: AB7-1	1280	1280	100%	0.0	99%
AB517932.1[3]	*Mortierella globalpina* gene for *28S* ribosomal RNA, partial sequence	1269	1269	100%	0.0	99%

*Bolded text are hits from *M. alpina*
NCBI: National Center for Biotechnology Information
[1]Tsuji M., et al., 2016. Unpublished, submitted to NCBI
[2](Schoch et al. 2012)
[3]Hirose D, et al., 2009. Unpublished, submitted to NCBI

Due to the high degree of identity shared among the hits of the BLAST hits for *18S* and *26S* rRNA, these sequencing results confirm that the organism used by Hubei Fuxing to produce ARA-rich oil is *M. alpina*.

D. PRODUCTION PROCESS

ARA-rich oil is produced at Hubei Fuxing Biotechnology Co., Ltd in Hanchuan, China. Hubei Fuxing has been certified as meeting the Food Safety System Certification (FSSC) 22000 standards; therefore, Hubei Fuxing manufactures the ARA-rich oil in accordance with current Good Manufacturing Practices (cGMP).

1. Production of ARA-Rich Oil

The production of ARA-rich oil occurs in the following steps: expansion of a pure *M. alpina* AF culture, collection and extraction of the crude oil, and refining of the crude oil into the final product. One production period is considered one batch.

All culturing steps in the generation of ARA-rich oil occur under aerobic conditions in the absence of light in a closed system. Each culture step is monitored for microbial contamination by light microscopy to confirm healthy cell morphology and cell population with no evidence of microbiological contamination before advancing to the next step for producing ARA-rich oil. All media are sterilized. Prior to initiation and culturing of *M. alpina* AF, all raw materials and food contact materials are inspected and must comply with internal quality parameters as a critical control point.

a. Initiation of M. alpina AF culture

M. alpina AF is maintained as spores, stored in liquid nitrogen. A solid slant culture is maintained as a stock for future cultures, and the genotypic identity of the strain is validated every year.

To initiate a culture from spores, spores are transferred to a shake flask with sterile culture medium for inoculation. The culture is monitored for the absence of microbial contamination and the cell density is assessed as an indicator of a healthy population. The culture is considered ready for expansion to the seed tank when the biomass meets cell density quality control specifications.

b. Expansion of M. alpina AF culture

The initiation biomass is divided equally and transferred into four Level 1 seed tanks with stirring and aeration until the biomass meets the cell density quality control specification. The biomass from the Level 1 seed tank is then equally distributed into four Level 2 seed fermentation tanks and cultured until the biomass meets cell density quality control specifications. The entire culture is then divided and transferred to eight Level 3 fermentation tanks to promote arachidonic

acid production. The Level 3 fermentation step is complete when the biomass, total oil, and ARA content internal control standards for the semi-finished product are met. The ARA content of the biomass in the Level 3 fermentation tank is a critical control point for the production process. The expansion process for *M. alpina* AF is shown in Figure 1.

The fermentation steps are controlled to ensure consistent pH, aeration rate, and temperature. All culture steps are monitored for evidence of microbiological contamination and cell division as an indicator of a viable fungal population every 8 hours. If any visible microbiological contamination is detected by light microscopy, the entire biomass is discarded via sewage disposal.

c. Crude oil extraction

After the biomass in the fermentation tank has generated sufficient ARA content to meet internal quality control specifications, the biomass is filtered from the medium and the medium is discarded. The biomass is washed 3 times with sterilized water. The biomass is then dried with food grade commercial driers under controlled temperature and pressure such that the moisture of the dried biomass meets internal quality specifications. The crude oil is extracted with butane under controlled temperature and pressure conditions. After extraction, the fungal dregs of the biomass are discarded, and the butane is removed from the crude oil.

The solvent leaching process is repeated multiple times to remove the butane from the crude oil. The butane is reserved, refined, and reused for the next production. Due to the sustained high temperatures used in refining, and that butane is gaseous at room temperature, no butane is expected to be present after the crude oil is refined. Hubei Fuxing has set a product specification for residual solvents at less than 1 mg/kg to confirm that butane has been removed from the finished product. The crude oil extraction is shown in Figure 1.

The crude oil has no more than 5% biomass and the following quality attributes are recorded: peroxide, taste/smell, acid value, phosphorus, unsaponifiable matter, and anisidine value. The crude oil is stored in stainless steel barrels under a layer of N_2 to minimize oxidation at no more than -5^{o}C for an average of 2 months before refining. The storage time will not exceed 2 years. The crude oil is stored temporarily in a warehouse before continuing to ARA-rich oil refining in the next step.

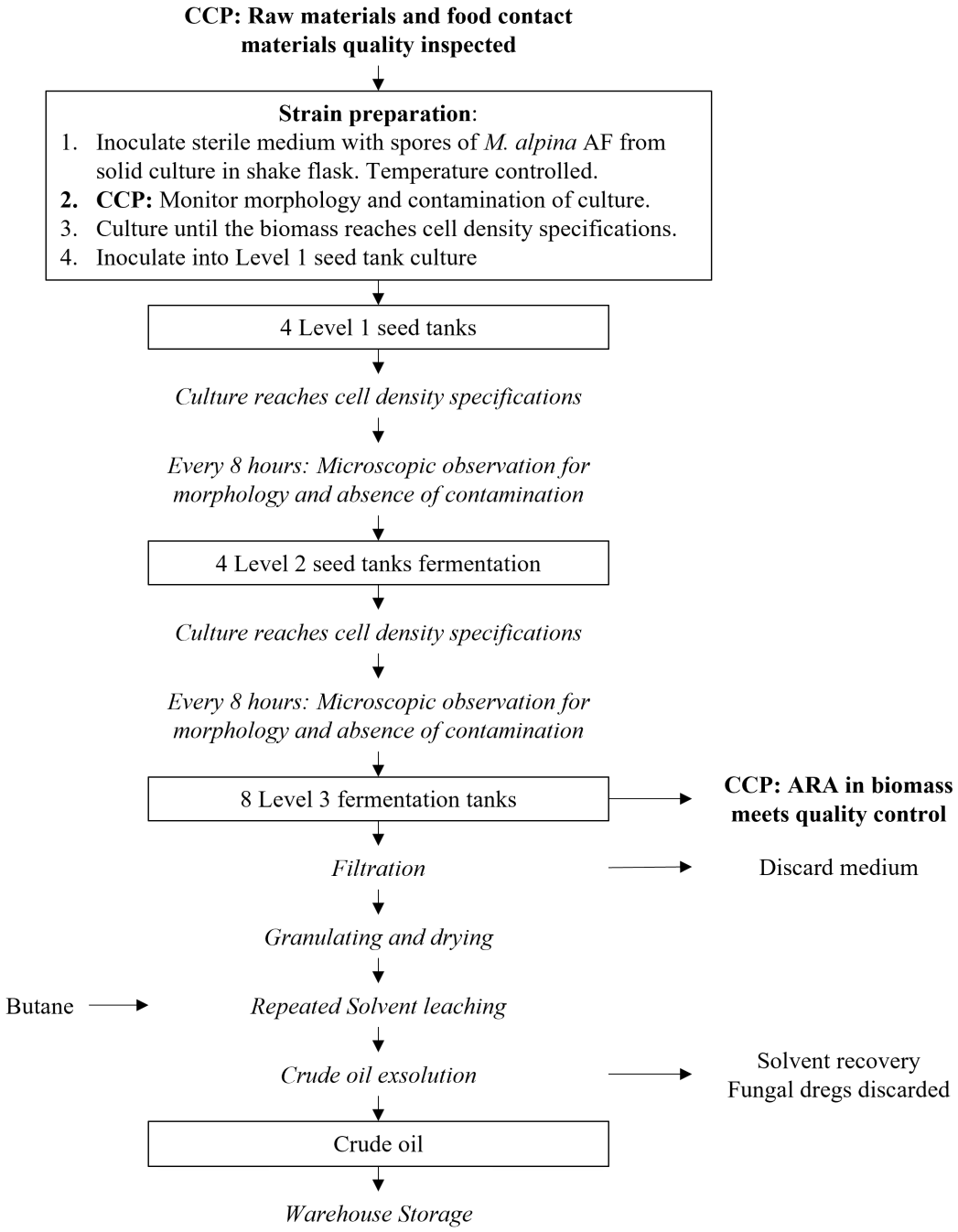


Figure 1. Production of ARA-rich Crude Oil

A *M. alpina* AF solid slant culture is used to inoculate four Level 1 seed tanks. After inoculation, *M. alpina* AF is cultured until the biomass reaches density quality control specifications. All cultures are monitored for microbiological contamination and normal cell morphology. The biomass from the Level 1 tank is used to inoculate the four Level 2 tanks. The biomass is cultured until it reaches density quality control specifications. The biomass is then used to inoculate eight Level 3 fermentation tanks for the final culture step. After culturing in the fermentation tank, a sample is taken to measure ARA before producing the crude oil. Fermentation is finished when sufficient ARA content is measured in the biomass. Then the biomass is filtered from the medium, dried, and butane is used to extract the crude oil. *CCP: critical control point*

d. ARA-rich oil refining

The oil refining process is shown in Figure 2. The refining process begins with the precipitation of phospholipids and free fatty acids from the crude ARA-rich oil through the addition of purified water and citric acid. The phospholipid content must comply with internal quality specifications by the end of this step and is controlled as a critical control point. All waste materials are discarded via sewage disposal. The oil is then deacidified with purified water and sodium hydroxide prior to washing. The acid value must comply with internal quality specifications and is controlled as a critical control point. Next, the crude oil is washed with water to remove saponins and residual soaps. Nitrogen, activated carbon, and activated clay are used to further refine the oil in the decoloration step. Nitrogen is used during the refining process to prevent oxidation of the oil. Following decoloration, the activated carbon and clay are removed by filtration. The oil is deodorized by steam, and a critical control point is in place to control the peroxide value of the oil. The deodorized oil is then filtered, and antioxidants (Vitamin E and ascorbyl palmitate) are added to the finished ARA-rich oil. The finished ARA-rich oil is then subjected to quality control by testing for compliance with the product specifications.

The critical control points ensure the quality and consistency of the oil by controlling phospholipids, acid value, and peroxide value during the refining process. The final product is reworked if it fails to meet product specifications for ARA-rich oil and may re-enter the production process as needed to meet the quality requirements for the finished oil.

Finished ARA-rich oil is stored in food-grade aluminum bottles at -10°C for an average of three months until delivered to the customer. The finished ARA-rich oil is stored for no more than 14 months.

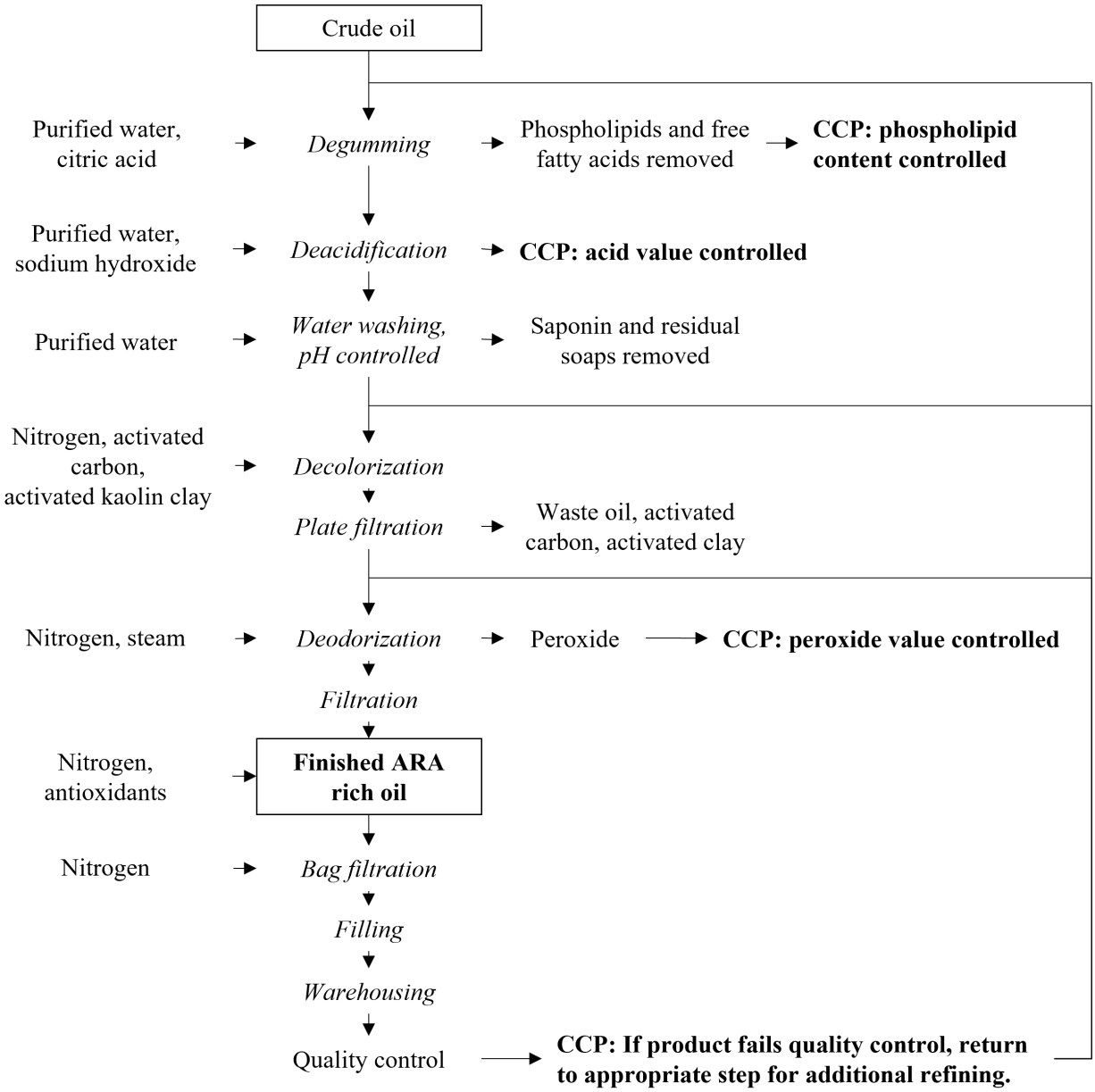


Figure 2. Refining of Crude Oil to Yield Final Product, ARA-rich Oil

The crude oil is refined via degumming, deacidification, water processing, decolorization, and deodorization to generate the final product, ARA-rich oil. *CCP: critical control point*

2. Raw Materials, Processing aids, and Food Contact Substances

All processing aids used in the production of ARA-rich oil comply with Title 21 of the Code of Federal Regulations (21 CFR), and/or Food Chemicals Codex (FCC) specifications. All raw materials used in the culture of *M. alpina* AF are food grade materials that comply with 21 CFR. Similarly, all aluminum bags and drums comply with food grade acceptance criteria. These processing aids and their role in the production of ARA-rich oil are described below in Table 3.

Table 3. Processing Aids and Raw Materials Used in the Manufacture of ARA-Rich Oil

Processing Aid	Role in Production	US Regulations
Oral Glucose/Dextrose, monohydrate	Fermentation	21 CFR §168.111
Yeast powder/dry yeast	Fermentation	21 CFR §172.896
Yeast extract	Fermentation	21 CFR §184.1983
Potassium dihydrogen phosphate	Fermentation	No 21 CFR citation, complies with FCC monograph
Butane	Extraction	21 CFR §184.1165
Sodium Carbonate	Fermentation	21 CFR §184.1742
Sodium Hydroxide	Oil Refining	21 CFR §184.1763
Citric Acid, monohydrate	Oil Refining	21 CFR §184.1033
L-Ascorbyl palmitate	Anti-oxidant	21 CFR §182.3149
Activated clay, kaolin	Oil Refining	21 CFR §186.1256
Activated Carbon	Oil Refining	No 21 CFR citation, complies with FCC monograph
Polyethersulfone	Filter	21 CFR §177.2440
Vitamin E oil (RRR-Tocopherol concentrate, mixed)	Anti-oxidant	21 CFR §184.1890
Polytetrafluoretheylene	Filter	21 CFR §177.1550
Aluminum bottles	Storage	FDA issued favorable opinion on the acceptability for food contact use without a premarket approval requirement (FDA memo 2012)

CFR: Code of Federal Regulations
FCC: Food Chemicals Codex

E. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. Product Specifications

To ensure a consistent food-grade product, Hubei Fuxing tests each batch of ARA-rich oil for compliance with a defined set of product specifications (Table 4). These parameters are assessed by the compendial methods at an ISO/IEX 17025: 2005-accredited laboratory. Guobiao (GB) Chinese national standard methods issued by the Standardization Administration of China and are based on American Oil Chemists' Society (AOCS) methods or International Organization for Standardization (ISO) methods. Data from three batches of ARA-rich oil demonstrate control of the production process and compliance with the product specifications.

Table 4. Product Specifications and Batch Data for Three Batches of Hubei Fuxing's ARA-Rich Oil

Parameter	Specification	Method	LOQ	Batch Number		
				A19050301J	A19050501J	A19050701J
Physical/Chemical Parameters						
Appearance	Pale to dark yellow oil	GB/T 5525-2008	-	Pass	Pass	Pass
Taste & Smell	Characteristic taste and smell	GB/T 5525-2008	-	Pass	Pass	Pass
25.4 mm color	Y ≤ 35, R ≤ 5	GB/T 22460-2008	-	Y=3.0, R=0.5	Y=2.5, R=0.5	Y=2.4, R=0.5
ARA (C20:4n6), %	≥ 40	GB 5009.168-2016 Third Method	0.02	45.7	45.9	44.8
Moisture, g/100g	≤ 0.05	GB 5009.236-2016	0.01	0.03	0.03	0.02
Impurities and Contaminants						
Acid value, mg KOH/g	≤ 0.5	GB 5009.229-2016	0.05	0.24	0.25	0.24
Trans fatty acid (%)	≤ 1.0	GB 5009.257-2016	0.02	0.29	0.33	0.32
Free fatty acids, % oleic acid	≤ 0.2	ISO 660:2009	0.05	0.12	0.12	0.12
Insoluble impurity (%)	≤ 0.2	GB/T 15688-2008	-	0.02	0.02	0.02
Unsaponifiable matter, %	≤ 3.0	GB/T 5535.2-2008	0.1	0.99	0.95	0.99
p-Anisidine value	≤ 10	GB/T 24304-2009	-	5.5	5.2	6.5
Peroxide value, meq/kg	≤ 2.0	GB 5009.227-2016 First Method	0.0006	ND	ND	ND
Residual solvent (mg/kg)	≤ 1.0	GB 5009.262-2016	1	ND	ND	ND
Metal Contaminants						
Mercury (Hg), mg/kg	≤ 0.01	GB 5009.17-2014 First Chapter second method	0.005	ND	ND	ND
Lead (Pb), mg/kg	≤ 0.1		0.05	ND	ND	ND
Arsenic (As), mg/kg	≤ 0.1		0.05	ND	ND	ND
Cadmium (Cd), mg/kg	≤ 0.1		0.01	ND	ND	ND
Copper (Cu), mg/kg	≤ 1.0		0.1	ND	ND	ND
Microbial and Other Contaminants						
Coliforms, cfu/mL	≤ 3	GB 4789.3-2016 Second method	-	<1.0	<1.0	<1.0
Molds, cfu/mL	≤ 10	GB 4789.15-2016 First method	-	<1.0	<1.0	<1.0
Yeast, cfu/mL	≤ 10	GB 4789.15-2016 First method	-	<1.0	<1.0	<1.0
Salmonella, /25g	Negative	GB 4789.4-2016	-	ND	ND	ND
Aerobic plate count, cfu/mL	≤ 1000 CFU/g	GB 4789.2-2016	-	<1.0	<1.0	<1.0
Aflatoxin B1 (μg/kg)	ND	DIN EN 14123, mod.	0.1	ND	ND	ND
Cronobacter sakazakii	ND in 100 mL	GB 4789.40-2016	0.3 MPN/100 mL	ND	ND	ND

Abbreviations used:
GB: mandatory Chinese national standard issued by the Standardization Administration of China; GB/T: recommended Chinese national standard issued by the Standardization Administration of China; ND: not detected; LOQ: Limit of quantitation; meq: milliequivalents; mod.: modified; CFU: colony forming units; DIN EN: Deutches Institut für Normang (German Institute for Standardization) European Standards; MPN: most probable number.

2. Other Quality Attributes

To characterize the quality of ARA-rich oil, Hubei Fuxing quantified the levels of fatty acids and sterols in the finished product.

a. Percentage of fatty acids in ARA-rich oil

To evaluate the variability in the levels of individual fatty acids in ARA-rich oil, Hubei Fuxing quantified the fatty acids in three batches of the finished product. Arachidonic acid is the predominant fatty acid present in the ARA-rich oil (Table 5). This testing is performed on every batch of ARA-rich oil.

Table 5. Percentage Fatty Acids in ARA-Rich Oil

Fatty Acids (%)	Batch Number		
	A19050301J	A19050501J	A19050701J
C4:0 Butyric Acid	ND	ND	ND
C6:0 Caproic Acid	ND	ND	ND
C8:0 Caprylic Acid	ND	ND	ND
C10:0 Capric Acid	0.03	0.02	0.03
C11:0 Hendecanoic Acid	ND	ND	ND
C12:0 Lauric Acid	ND	ND	ND
C13:0 Tridecaonic Acid	ND	ND	ND
C14:0 Myristic Acid	0.46	0.41	0.44
C14:1 Myristoleic Acid	ND	ND	ND
C15:0 Pentadecanoic Acid	0.15	0.14	0.13
C15:1 Pentadecenoic Acid	ND	ND	ND
C16:0 Palmitic Acid	9.61	9.63	9.85
C16:1 Palmitoleic Acid	0.3	0.22	0.26
C17:0 Margaric Acid	0.33	0.33	0.3
C17:1 Margaroleic Acid	ND	ND	ND
C18:0 Stearic Acid	6.27	6.32	6.53
C18:1 Oleic Acid	5.42	5.3	5.66
C18:1(n-9) Elaidic Acid	0.10	0.11	0.10
C18:2 Linoleic Acid	7.75	7.18	8.48
C18:2(n-6) Linoleaidic Acid	0.19	0.22	0.21
C18:3(n-3) Alpha-Linolenic Acid	0.52	0.44	0.59
C18:3(n-3) Trans-Linolenic Acid	ND	ND	ND
C18:3(n-6) Gamma-Linolenic Acid	2.81	2.81	2.78
C20:0 Arachidic Acid	ND	0.81	0.78
C20:1 Gondoic Acid	0.33	0.34	0.35
C20:2 Eicosadienoic Acid	0.64	0.66	0.63
C20:3(n-3) Eicosatrienoic Acid	0.12	0.11	0.11
C20:3(n-6) Eicosatrienoic Acid	5.01	5.65	5.22
C20:4 Eicosatetraenoic (Arachidonic) Acid	**45.70**	**45.90**	**44.80**
C20:5(n-3) Eicosapentaenoic Acid	0.28	0.24	0.27
C21:0 Heneicosanoic Acid	0.07	0.06	0.06
C22:0 Behenic Acid	2.91	2.92	2.90
C22:1 Erucic Acid	0.07	0.07	0.07
C22:2(n-6) Docosadienoic Acid	0.05	0.05	0.05

Table 5. Percentage Fatty Acids in ARA-Rich Oil			
Fatty Acids (%)	**Batch Number**		
	A19050301J	**A19050501J**	**A19050701J**
C22:6(n-3) Docosahexaenoic Acid	0.30	0.04	ND
C23:0 Tricosanoic Acid	0.06	0.06	0.05
C24:0 Lignoceric Acid	9.38	9.66	9.13
C24:1 Nervonic Acid	0.30	0.31	0.30
Mono-unsaturated fatty acids total	6.51	6.37	6.74
Omega-3 fatty acids	1.21	0.82	0.97
Omega-6 fatty acids	62.20	62.40	62.10
Omega-9 fatty acids	6.22	6.14	6.48
Poly-unsaturated fatty acids total	63.40	63.30	63.10
Saturated fatty acids total	30.10	30.40	30.10
Total EPA + DHA Omega-3 fatty acids	0.57	0.28	0.27
Total of trans fatty acids	0.29	0.33	0.32
Total omega fatty acids	69.60	69.4	69.60
Method: GB 5009.168-2016 Third Method, performed by Eurofins Tech. Service, Suzhou 215000, Jiangsu Province, P.R. China Limit of Quantitation: 0.02%			

b. Sterols in ARA-rich oil

Three batches of ARA-rich oil were assessed for the presence of sterols, including desmosterol, which is present in human milk and known to be produced by *M. alpina* (Table 6) (Shimizu et al. 1992; Nes and Nichols 2006; van Brakel et al. 2022). Although there is no validated method for the quantitation of sterols in oils derived from *M. alpina*, the sterols were quantified in an ISO/IEX 17025: 2005-accredited laboratory using internal gas chromatography-flame ionization detector/mass spectrometry (GC-FID/MS), which is the same method used to quantify sterols in GRN 963 (2021). In brief, the unsaponifiable matter of the subject of this Notice was isolated by solid phase extraction on an aluminum oxide column. The sterol fraction was then separated by gas chromatography, coupled with a flame ionization detector. The identification of sterol residues was confirmed by mass spectrometry and quantified with the use of an internal standard.

The most abundant sterols were ergosta 5,24(25) dienol, also known as 24-methyl desmosterol (https://pubchem.ncbi.nlm.nih.gov/compound/193567#section=2D-Structure), and desmosterol, both of which are produced by *M. alpina* (Shimizu et al. 1992; Nes and Nichols 2006). The sterol listed as "Isomer of 24 methylene cholesterol" is a predicted isomer based on the spectral data, but is considered hypothetical as it was not associated with a known standard. Isomer of 24-methylene cholesterol, 24-methylene cholesterol, beta-sitosterol, zymosterol, and cholesterol are also present in the subject of this Notice, but at lower levels than 24-methyl desmosterol and desmosterol. The unknown sterols in the subject of this GRAS Notice account for less than 5% of the total sterol content and correspond to peaks in the spectra that are too weak to be successfully identified by the GC-FID/MS.

Table 6. Sterol Content of ARA-rich oil			
Sterol (% of sterols)	**Batch Number**		
	A21110301J	**A21110601J**	**A21110901J**
Ergosta 5,24(25) dienol (synonym for 24-Methyldesmosterol)	54.1%	57.3%	52.5%
Desmosterol	20.5%	18.3%	18.2%
Isomer of 24 methylene cholesterol*	13.0%	11.8%	14.8%
24 methylene cholesterol	6.2%	6.0%	7.5%
Beta sitosterol	1.4%	1.5%	1.3%
Zymosterol	0.9%	1.1%	0.8%
Cholesterol	0.2%	0.2%	0.2%
Unknown No. 1	1.3%	0.9%	1.0%
Unknown No. 2	0.5%	0.5%	0.6%
Unknown No. 3	0.5%	0.7%	0.9%
Unknown No. 4	1.4%	1.7%	2.3%
Sum of unknown peaks	3.7%	3.8%	4.8%
Sterol Content	10664 mg/kg	9875 mg/kg	11601 mg/kg
Limit of Quantitation: 10 mg/kg N.D.: not detected *Hypothetical isomer			

c. Glycidyl and 3-Monochloro-propanediol (MCPD)-esters 1,2-diol Fatty Acid Esters in ARA-rich oil

Esters of 3-monochloro-propanol-1,2 diol (3-MCPD) and glycidol may be contaminants of processed vegetable oils that are formed during oil refining when the oils are treated with hydrochloric acid at high temperatures and pressure. Although Hubei Fuxing does not use hydrochloric acid in the manufacturing process, glycidyl and 3-MCPD fatty acid esters were quantified in three batches of ARA-rich oil from *M. alpina* AF for due diligence purposes by SGS-CSTC Standards Technical Services Co., Ltd, which is an ISO/IEX 17025: 2005-accredited lab using a gas chromatography-mass spectrometry-based compendial method (Table 7). Glycidyl fatty acid esters were below the limit of quantitation in all three batches. 3-Monochloro-propanol-1,2-diol fatty acid esters were detected in all batches with mean concentration of 0.28 ± 0.02 mg 3-MCPD/kg ARA-rich oil and a maximum of 0.3 mg 3-MCPD/kg ARA-rich oil. Although there are no limits for glycidyl and 3-MCPD fatty acid esters in the United States, glycidyl and 3-MCPD fatty acid esters must not exceed the maximum levels of 6 and 15 µg/kg, respectively, for liquid infant and follow-on formula in the European Union (Commission Regulation (EU) 2020/1322).

Per 21 CFR 107.100, infant formulas must contain a maximum of 6.0 g fat/100 kcal. Assuming that term infant formulas contain approximately 670 kcal/L (Martinez and Ballew, 2011), the target ARA concentration in term infant formulas is 0.75 % total fat, and ARA-rich oil from *M. alpina* AF contains not less than 40% ARA and a maximum amount of 0.3 mg/kg 3-MCPD fatty acid esters, the resulting amount of 3-MCPD fatty acid esters in infant formula will

be approximately 0.23 µg/kg formula, which is orders of magnitude below the maximum level of 15 µg/kg established in the European Union. Additionally, if glycidyl fatty acid esters were present at the limit of quantitation for the assay, the resulting level in infant formula will be 0.077 µg/kg formula, which is also orders of magnitude below the limit of 6 µg/kg established in the European Union.

Table 7. 3-Monochloro-propanol-1,2 diol (MCPD)-esters and Glycidyl Esters in ARA-rich oil

Parameter	Method	LOQ	Batch No		
			A22080201H	A22080601H	A22080901H
Sum of free 3-MCPD, 3-MCPD-ester, detected as free 3-MCPD	AOCS Official Method Cd 29b-13 GC-MS	0.1 mg/kg	0.29 mg/kg	0.26 mg/kg	0.30 mg/kg
Sum of free glycidol, glycidylester detected as free glycidol	AOCS Official Method Cd 29b-13 GC-MS	0.1 mg/kg	N.D.	N.D.	N.D.

MCPD: monochloro-propanol-1,2 diol
LOQ: limit of quantitation
N.D.: not detected

F. STABILITY OF ARA-RICH OIL

The stability of three batches of ARA oil was investigated under ambient conditions (25ºC, humidity 75%) for 14 months. The ARA-rich oil was stored in aluminum bottles, the commercial packaging for the product, and flushed with nitrogen gas to minimize oxidation. Samples were tested using the same methods as described in Table 4 at 0, 1, 2, 4, 6, 8, 10, 12, and 14 months after packaging. The results are shown in Table 8, demonstrating compliance with product specifications up until 12 months. The three batches complied with the product specifications at 14 months, except for the Acid Value of batch A19050501J, which was out of specification at the 14-month time point. Together, the results of this study support a shelf life of 12 months when stored after the manufacture date under ambient conditions.

Table 8. Stability of ARA-Rich Oil

Parameter	Specification	Time (months)								
		0	1	2	4	6	8	10	12	14
Batch A19050301J										
Taste & Smell	With its characteristic smell, no other peculiar smell found	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Acid Value (mg KOH/g)	≤ 0.5	0.24	0.25	0.29	0.34	0.34	0.36	0.45	0.47	0.49
Peroxide Index (meq/kg)	≤ 10	0	0.10	0.26	0.50	0.95	1.12	1.25	1.47	2.22
ARA (%)	≥ 40	45.71	45.65	44.98	44.78	44.34	44.26	44.20	43.10	43.02
p-Anisidine value	≤ 10	5.5	5.7	6.4	7.2	7.7	8.3	8.7	9.2	9.4
Coliforms (cfu/mL)	≤ 3	ND	ND	ND	ND	ND	ND	ND	ND	ND
Molds (cfu/mL)	≤ 10	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yeast (cfu/mL)	≤10	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella	Negative in 25 g	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Aerobic Plate Count (APC) (cfu/mL)	≤ 1000	ND	ND	ND	ND	ND	ND	ND	ND	ND
Batch A19050501J										
Taste & Smell	With its characteristic smell, no other peculiar smell found	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Acid Value (mg KOH/g)	≤ 0.5	0.25	0.25	0.28	0.36	0.36	0.38	0.44	0.48	0.51
Peroxide Index (meq/kg)	≤ 10	0	0.19	0.33	0.55	1.10	1.21	1.33	1.76	2.05
ARA (%)	≥ 40	45.90	45.75	45.28	45.08	44.84	44.46	44.20	44.05	43.97
p-Anisidine value	≤ 10	5.2	5.6	6.2	7.0	7.4	8.5	8.7	9.0	9.6
Coliforms (cfu/mL)	≤ 3	ND	ND	ND	ND	ND	ND	ND	ND	ND
Molds (cfu/mL)	≤ 10	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yeast (cfu/mL)	≤10	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella	Negative in 25 g	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Aerobic Plate Count (APC) (cfu/mL)	≤ 1000	ND	ND	ND	ND	ND	ND	ND	ND	ND
Batch A19050701J										
Taste & Smell	With its characteristic smell, no other peculiar smell found	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass	Pass
Acid Value (mg KOH/g)	≤ 0.5	0.24	0.26	0.28	0.32	0.36	0.37	0.40	0.42	0.48
Peroxide Index (meq/kg)	≤ 10	0	0.27	0.34	0.68	1.07	1.26	1.41	1.65	1.83
ARA (%)	≥ 40	44.81	44.62	44.38	44.18	44.04	43.76	43.50	43.33	43.18
p-Anisidine value	≤ 10	6.5	6.9	7.4	7.8	8.2	8.6	9.0	9.3	9.5
Coliforms (cfu/mL)	≤ 3	ND	ND	ND	ND	ND	ND	ND	ND	ND
Molds (cfu/mL)	≤ 10	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yeast (cfu/mL)	≤10	ND	ND	ND	ND	ND	ND	ND	ND	ND
Salmonella	Negative in 25 g	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Aerobic Plate Count (APC) (cfu/mL)	≤ 1000	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND: not detected; neg: negative
cfu: colony forming units
meq: milliequivalents
Storage Conditions: 25°C, humidity 75%

III. DIETARY EXPOSURE

The subject of this GRAS Notice is intended to be used as a source of ARA in cow's milk-based and soy-based exempt infant formula for pre-term infants and non-exempt infant formula for term infants. The subject of this GRAS Notice is also intended to be used as a substitute for the *M. alpina*-derived ARA-rich oils that are the subjects of GRN 326 and GRN 963, which received "no questions" letters from the FDA in 2010 and 2021, respectively. The use levels and resulting exposure for the subject of this GRAS Notice are equivalent to those described for GRN 326 and GRN 963 and are incorporated by reference (GRN 326, 2010, page 60, stamped page 70; GRN 963, 2021, page 22).

A. INTENDED EFFECT

ARA-rich oil is intended to be used as a source of ARA, a fatty acid naturally present in human milk and known to play a role in infant development. Brenna et al. (2007) conducted a meta-analysis of ARA concentrations in mature human milk based on published data from 65 studies spanning 1986 to 2006 and involving 2474 women worldwide. The mean and standard deviation of ARA concentration as a percentage of total fatty acids was 0.47% ± 0.13% (range: 0.24-1.0%). This evaluation reveals a broad range of ARA levels in human milk on a worldwide basis and shows the range of possible infant exposure to ARA, which provides a guide for levels of ARA supplementation in infant formulas.

Based on scientific consensus and the current knowledge regarding the importance of LCPUFA in infant nutrition, their presence in human milk, and the guidelines and recommendations established by the European Academy of Paediatrics, World Association of Perinatal Medicine, the Early Nutrition Academy, and the Child Health Foundation, infant formula should contain 0.3 to 0.5 weight percent of total dietary fat, with the minimum amount of ARA being equivalent to the DHA content (Koletzko et al., 2014; Koletzko et al., 2020).

B. HISTORY OF USE

Fungal oils as a source of ARA have been used in commercially available infant formulas in at least 50 countries since the early 1990s. In the United States, ARA-rich oil generated from *M. alpina* has been the subject of multiple GRAS notifications: GRN 963 (2021); GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001). The information provided in GRN 94 for ARA-rich oil (SUNTGA40S), and in the published opinion on the novel food authorization for this oil (EFSA, 2008), noted that the production of long chain PUFAs by microorganisms, including *M. alpina,* has been employed for several years. Food Standards Australia New Zealand concluded that ARA-rich oil was a safe source of ARA for use in infant formula in 2003 (FSANZ 2003).

C. INTENDED USE

The intended use of the subject of this GRAS Notice is to provide a source of ARA in term and preterm infant formulas at a concentration consistent with that of human milk and the current guidelines regarding safe infant feeding practices. The ARA content of human milk varies from 0.34-1.22% of total fatty acids among different populations. The proposed use of ARA-rich oil is to provide 0.75% and 0.40% ARA by weight of fatty acids in term and pre-term infant formulas, respectively, which is equivalent to 1.875% of total fat in non-exempt term infant formula and 1.0% of total fat in exempt pre-term infant formula. Importantly, these levels are consistent with those recommended by the European Academy of Paediatrics and the Child Health Foundation. Additionally, the subject of this GRAS Notice will be used in formulas for term and pre-term infants with a safe and suitable source of DHA at levels that comply with current recommendations and guidelines (Koletzko et al., 2008; Koletzko et al., 2014; Koletzko et al., 2020; Koletzko and Lapillonne, 2021).

D. ESTIMATED DAILY INTAKE

An estimate of exposure to ARA from its intended use is based on target ARA concentrations of 0.75% and 0.40% of total fat in term and pre-term infant formula, respectively. The assumptions upon which this estimation is made are the same as those cited in GRN 326, pg 60 and GRN 963, pg 22 (FDA, 2010; FDA, 2021). Assuming human infants consume approximately 100 kcal/kg body weight/day (term infants) to 120 kcal/kg body weight/day (pre-term infants), of which fat comprises about 50% of those calories, an infant will consume about 5.6 g (term infants) to 6.7 g (pre-term infants) of fat/kg body weight/day (1 g fat = 9 kcal). These correspond to intakes of ARA of 42 mg and 27 mg ARA/ kg body weight/day (corresponding to 105 and 67 mg of ARA-rich oil/kg body weight/day) for term infants and pre-term infants, respectively.

IV. SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with the ARA-rich oil ingredient.

V. COMMON USE IN FOOD BEFORE 1958

The statutory basis for the conclusion of GRAS status of ARA-rich oil derived from *M. alpina* AF in this document is not based on common use in food before 1958. The GRAS Notice is based on scientific procedures.

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The FDA has issued 'no questions' letters for six GRAS notices for ARA-rich oils derived from *M. alpina* for use in infant formula: GRN 41 (2001), GRN 80 (2001), GRN 94 (2006), GRN 326 (2010), GRN 730 (2018), and GRN 963 (2021). The safety of the ingestion ARA-rich oils from *M. alpina* is supported by in vitro studies, in vivo animal studies, and clinical studies in term and preterm infants. Studies include absorption, distribution, metabolism, and excretion (ADME); acute and subchronic toxicology; reproductive and developmental toxicology; and mutagenicity and genotoxicity, along with clinical and epidemiological studies. Kroes et al. (2003) and Kremmyda et al. (2011) have reviewed and summarized the well-understood metabolism of dietary ARA, which is similar to other dietary fatty acids.

The ARA-rich oil from Hubei Fuxing is compositionally similar to the ARA-rich oils described in the previously mentioned GRAS Notices based on comparisons of the manufacturing process and finished product specifications. Therefore, the information and data provided in the other GRAS notices that received "no questions" letters from the Agency also support the safety of the ARA-rich oil that is the subject of this GRAS Notice. The GRAS notices cited provide documentation of the consensus of opinion based on publicly available information that establishes there is reasonable certainty of no harm to target consumers from ingesting ARA-rich oil from the intended uses and use levels. Hubei Fuxing Biotechnology Co. concludes that ARA-rich oil is GRAS when used as an ingredient in term and preterm infant formula at the intended use levels.

This Notice incorporates by reference the safety and metabolism studies discussed in previous GRNs (GRN 963: pages 25-33; GRN 730: pages 28–47; GRN 326: pages 61-153; GRN 94: pages 78-318; GRN 80: stamped pages 16-23 and 48-55; GRN 41: stamped pages 108-118 and 175-418) and will not discuss previously reviewed references in detail.

A. COMPOSITIONAL EQUIVALENCE AMONG ARA-RICH OILS FROM *M. ALPINA* THAT ARE GRAS

All ARA-rich oils derived from *M. alpina* that received "no questions" letters from the FDA have similar product specifications for ARA content, oil quality parameters (e.g., peroxide value, acid value, moisture), fatty acid profiles, and sterol profiles and are therefore essentially equivalent. The ARA-rich oil produced by Hubei Fuxing also complies with the impurity specifications listed in the Food Chemicals Codex (FCC) monograph for ARA from fungal (*Mortierella alpina*) oil.

1. Product Specifications Comparison Among ARA-rich Oils from *M. alpina* that are GRAS

Although some of the specifications for Hubei Fuxing's ARA-rich oil are more stringent than the specifications for other ARA-rich oils derived from *M. alpina* that are GRAS and received "no questions" letters from the FDA (e.g. acid value, anisidine value, mercury, and moisture), a comparison among these product specifications demonstrates that these oils are essentially equivalent (Table 9). It is, therefore, reasonable to conclude that the safety of the subject of this GRAS Notice is equivalent to the safety of the ARA-rich oils that are the subjects of GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), and GRN 80/41 (2001).

Table 9. Specifications of Hubei Fuxing's ARA-Rich Oil Compared with Previous GRAS Notices for ARA-rich oil from *M. alpina*

Parameter	**Hubei Fuxing's ARA-rich oil**	GRN 963 (2021)	GRN 730 (2018)	GRN 326 (2010)	GRN 94 (2006)	GRNs 80 and 41* (2001)
ARA, C20:4n6, weight%	**≥ 40**	≥ 40[a]	≥ 40	≥ 40	≥ 40	38-44[b]
Acid value, mg KOH/g	**≤ 0.5**	≤ 1.0	≤ 0.5	≤ 1.0	N.S.	N.S.
Free fatty acids, % oleic acid	**< 0.2**	≤ 0.45	< 0.1[c]	≤ 0.2	≤ 0.2	≤ 0.4
Trans fatty acids, sum (area-%)	**N.S.**	≤ 1.0	≤ 1.0	N.S.	N.S.	≤ 1.0
Unsaponifiable matter, %	**≤ 3.0**	≤ 3.0	≤ 1.5	≤ 3.0	≤ 1.0	≤ 3.5
Anisidine value	**≤ 10**	≤ 20	≤ 10	≤ 20	N.S.	N.S.
Peroxide value, meq/kg	**≤ 2.0**	≤ 4.0	≤ 2.5	≤ 2.0	< 5.0	< 5.0
Residual solvents (Butane or Hexane), mg/kg	**≤ 1.0**	N.S.	N.S.	≤ 1.0	N.S.	N.S.
Mercury (Hg), mg/kg	**≤ 0.01**	≤ 0.01	≤ 0.05	≤ 0.05	< 0.5	< 0.2
Lead (Pb), mg/kg	**≤ 0.1**	≤ 0.02	N.S.	≤ 0.1	< 0.1	< 0.2
Arsenic (As), mg/kg	**≤ 0.1**	≤ 0.1	≤ 0.1	≤ 0.1	< 0.2	< 0.5
Cadmium (Cd), mg/kg	**≤ 0.1**	≤ 0.01	≤ 0.1	≤ 0.1	N.S.	N.S.
Moisture, g/100g	**≤ 0.05**	≤ 0.05	≤ 0.1	≤ 0.1	N.S.	N.S.
Coliforms, cfu/mL	**≤ 3**	N.S.	≤ 1	≤ 3	N.S.	N.S.
Molds, cfu/mL	**≤ 10**	N.S.	≤ 1	≤ 10	N.S.	N.S.
Yeast, cfu/mL	**≤ 10**	N.S.	≤ 1	≤ 10	N.S.	N.S.
Yeasts and molds, cfu/g	**N.S.**	≤ 100	N.S.	N.S.	N.S.	N.S.
Salmonella sp. /25g	**N.D.**	Negative	N.D.	N.S.	N.S.	N.S.
Aerobic plate count, cfu/mL	**< 1000**	≤ 1000[d]	< 100	N.S.	N.S.	N.S.
Cronobacter sakazakii	**N.D. in 100 mL**	N.S.	N.S.	N.S.	N.S.	N.S.
Enterobacteriaceae, /g	**N.S.**	Negative	N.S.	N.S.	N.S.	N.S.
Staphylococci coagulase pos., /g	**N.S.**	Negative	N.S.	N.S.	N.S.	N.S.
Pseudomonas aeruginosa, /g	**N.S.**	Negative	N.S.	N.S.	N.S.	N.S.

*GRN 41 and 80 are for the same product, ARASCO (arachidonic acid-rich single-cell oil), used at different levels in infant formula. GRN 80 expands the proposed use level in GRN 41.
[a]Specification listed as content arachidonic acid: ≥ 400 mg/g as triglyceride
[b]Specifications for other fatty acids are included.
[c]Free fatty acids measured as %oleic acids for GRN 730.
[d]Specification listed as total aerobic mesophilic count in cfu/g
N.S.: not specified; N.D.: not detected; cfu: colony forming units; meq: milliequivalents

2. Comparison of Hubei Fuxing ARA-rich oil with the Food Chemicals Codex Monograph for ARA from fungal (*Mortierella alpina)* oil

The specifications in the Food Chemicals Codex (FCC) monograph for ARA from fungal (*Mortierella alpina*) oil include ranges for twelve fatty acids on an area percent basis, as well as limits for ARA on a percent fatty acid basis, heavy metals, hexane residues, acid value, anisidine value, free fatty acids, peroxide value, and unsaponifiable matter (Food Chemicals Codex, 13th Edition). Hubei Fuxing's specifications include limits for ARA on a percent fatty acid basis, heavy metals, hexane residues, acid value, anisidine value, free fatty acids, peroxide value, and unsaponifiable matter. The product specifications for Hubei Fuxing's ARA-rich oil comply with the ARA specification on a percent fatty acid basis, the impurities specifications, and the quality attributes listed in the FCC monograph (Table 10). The mercury, acid value, and anisidine value product specifications for Hubei Fuxing's ARA-rich oil are more stringent than those described in the FCC monograph. Importantly, the subject of this GRAS Notice, as well as the subjects of the other GRAS Notices for ARA-rich oils derived from *M. alpina*, are intended to be used as a source of ARA, not sources of the other fatty acids listed in the FCC monograph for ARA from fungal (*Mortierella alpina*) oil. Consistent with this, the FCC monograph, the subject of this GRAS Notice, and the subjects of all of the other GRAS Notices for ARA-rich oils derived from *M. alpina,* all have specifications in place to ensure a minimum amount of ARA in the finished product.

With the exception of the subject of GRN 326, which was used to develop the FCC monograph, none of the subjects of the GRAS Notices for ARA-rich oils derived from *M. alpina* adhere to the area percent identity specifications for the twelve fatty acids listed in the FCC monograph (Table 11). Similarly, Hubei Fixing has not set specifications for individual fatty acids on an area percent basis because area percent is a qualitative measurement used to determine the abundance of a particular compound relative to the other compounds that are eluted from the column and detected. It does not quantitate the amount of each of the compounds in the oil. Therefore, the ranges for twelve fatty acids on an area percent basis specified in the FCC monograph are not relevant to defining the subject of this Notice as a source of ARA in infant formula. Additionally, the fat content of infant formulas is primarily made from vegetable oils, which contain the same twelve fatty acids specified in the FCC monograph, the fatty acid profile of the subject of this Notice is similar to the subjects of the other GRAS Notices for ARA-rich oils derived from *M. alpina* that received "no questions" letters (Table 10), and the subject of this Notice is intended to be used at 0.75% or 0.4% of the total fat in term infant formula or preterm infant formulas, respectively. Thus, the total dietary contribution of the individual fatty acid residues, other than ARA, from the subject of this Notice to the total fats provided in infant formula is negligible (Mendonca et al. 2017), and consistent with other GRAS Notices for ARA-rich oils derived from *M. alpina*.

Table 10. Comparison of Food Chemicals Codex 13th Edition ARA from Fungal (*Mortierella alpina*) Oil Monograph and Hubei Fuxing ARA-rich Oil Specifications

Parameter	FCC Limit	Hubei Fuxing ARA-rich oil Specification
Fatty Acid Composition, Area %		
Myristic Acid (14:0)	0.1-0.5%	N.S.
Palmitic Acid (16:0)	4.3-8.1%	N.S.
Palmitoleic Acid (16:1n-9)	0-0.4%	N.S.
Stearic Acid (18:0)	4.2-7.6%	N.S.
Oleic Acid (18:1n-9)	3.4-9.5%	N.S.
Linoleic Acid (18:2n-6)	3.8-15.2%	N.S.
gamma-Linolenic Acid (GLA) (18:3n-6)	1.7-2.74%	N.S.
Arachidic Acid (20:0)	0.6-1.0%	N.S.
Dihomo-gamma-linolenic acid (20:3n-6)	3.0-5.0%	N.S.
Arachidonic Acid (20:4n-6)	38.0-48.5%	N.S.
Behenic Acid (22:0)	2.5-4.1%	N.S.
Lignoceric Acid (24:0)	7.8-12.6%	N.S.
Assay, % total fatty acids		
ARA	NLT 38.0% ARA	≥ 40%
Impurities Specifications		
Arsenic	NMT 0.1 mg/kg	≤ 0.1 mg/kg
Cadmium	NMT 0.1 mg/kg	≤ 0.1 mg/kg
Lead	NMT 0.1 mg/kg	≤ 0.1 mg/kg
Mercury	NMT 0.1 mg/kg	≤ 0.01 mg/kg
Hexane Residues	NMT 1.0 mg/kg	≤ 1 mg/kg
Specific Tests		
Acid Value	NMT 1.0	≤ 0.5
Anisidine Value	NMT 20	≤ 10
Free Fatty Acids	NMT 0.2%	≤ 0.2%
Peroxide Value	NMT 2.0 mEq/kg	≤ 2.0 mEq/kg
Unsaponifiable Matter	NMT 3.0%	≤ 3.0%

NMT: not more than
NLT: not less than
N.S.: not specified

3. Fatty Acid Profiles Among ARA-rich oils From *M. alpina* that are GRAS

To confirm that the fatty acid content of the subject of this GRAS Notice is equivalent to the fatty acid content of the ARA-rich oils derived from *M. alpina* that are GRAS, the fatty acid profile for Hubei Fuxing ARA-rich oil was compared to the fatty acid profiles described in the ARA-rich oils presented in GRN 730 (2018), GRN 326 (2010), and GRN 94 (2006), all of which received "no questions" from the FDA (Table 11). The subject of this Notice and the subjects of GRN 730, GRN 326, and GRN 94 are not blended with other oils. The subjects of GRN 963 and GRN 41/80 were blended with high oleic sunflower oil to standardize ARA content and were not included in this comparison as these oils have a different fatty acid distribution than the other ARA-rich oils that are GRAS. The following fatty acids contribute over 86% of the total fatty acids in all ARA-rich oils, including Hubei Fuxing ARA-rich oil: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), eicosatrienoic (C20:3), arachidonic

(C20:4), behenic (C22:0), and lignoceric (C24:0) acids (indicated in bold text in Table 11). Stearic (C18:0), oleic (C18:1n9), linoleic (C18:2), linolenic (C18:3), arachidonic (C20:4), and behenic (C22:0) acids are present in similar amounts in all ARA-rich oils, with some differences observed in palmitic (C16:0) and linoleic (C18:2) acids. This comparison demonstrates that the fatty acid profile for Hubei Fuxing's ARA-rich oil is essentially equivalent to the fatty acid profiles described for GRN 730 (2018), GRN 326 (2010), and GRN 94 (2006).

Although there is variability in the amount of the specific fatty acids present in the oils, the differences are not expected to affect the safety of the ingredient because these fatty acids are naturally found in human milk, and the oils used to constitute the fat component of infant formula, and the diet (Floris et al. 2020; Mendonca et al. 2017; Orsavova et al. 2015). Moreover, considering the daily intake of fat by an infant, 5.6 g fat/kg/day for term infants and 6.7 g fat/kg/day for preterm infants, the dietary contribution of the fatty acid residues other than ARA from the subject of this Notice is negligible. Therefore, the differences in the amounts of the specific fatty acids are not expected to significantly affect the exposure to the individual fatty acid from the ingestion of ARA-rich oil in exempt or non-exempt infant formula.

Table 11. Fatty Acid Profile for Hubei Fuxing's ARA-rich oil and ARA-rich Oils that are GRAS				
Fatty Acid (%)	**Hubei Fuxing ARA-rich oil (n=3)**	**GRN 730 (n=3)**	**GRN 326 (n=5)**	**GRN 94 (n=3)**
Individual Fatty Acids				
C4:0 Butyric Acid	ND	-	-	-
C6:0 Caproic Acid	ND	ND	-	-
C8:0 Caprylic Acid	ND	ND	ND	-
C10:0 Decanoic Acid	0.027 ± 0.0058	ND	0.04 ± 0.0084	-
C11:0 Henedecanoic Acid	ND	-	-	-
C12:0 Lauric Acid	ND	ND	0.01 ± 0.0	-
C13:0 Tridecanoic Acid	ND	-	-	-
C14:0 Myristic Acid*	0.44 ± 0.025	0.39 ± 0.00058	0.26 ± 0.052	0.48 ± 0.10
C14:1-9c Myristoleic Acid	ND	ND	0.01 ± 0.0	TR
C15:0 10-Pentadecenoic Acid	0.14 ± 0.01	ND	0.086 ± 0.0089	0.17 ± 0.058
C15:1-10c	ND	ND	-	-
C16:0 Palmitic Acid*	**9.70 ± 0.13**	**7.26 ± 0.019**	**6.02 ± 0.65**	**13.8 ± 1.72**
C16:1-9c Palmitoleic Acid*	0.26 ± 0.04	0.12 ± 0.001	0.026 ± 0.0055	-
C16:1-7c	-	-	-	0.15 ± 0.05
C17:0 Margaric Acid	0.32 ± 0.017	0.24 ± 0.0015	0.18 ± 0.011	0.35 ± 0.071
C17:1-10c Heptadecenoic Acid	ND	0.078 ± 0.018	-	-
C18:0 Stearic Acid*	**6.37 ± 0.14**	**6.71 ± 0.013**	**5.11 ± 0.55**	**7.75 ± 0.22**
C18:1-7 Vaccenic Acid	-	0.29 ± 0.0069	-	0.4 ± 0.0
C18:1-9c Oleic Acid*	**5.46 ± 0.18**	**5.91 ± 0.017**	**4.97 ± 0.67**	**6.52 ± 0.18**
C18:1t Elaidic Acid	0.10 ± 0.01	-	-	-
C18:2-9c,12c Linoleic Acid*	**7.80 ± 0.65**	**6.02 ± 0.021**	**7.87 ± 0.75**	**10.9 ± 2.13**
C18:2t Linolelaidic Acid	0.21 ± 0.02	-	-	-
C18:3-6c,9c,12c γ-Linolenic Acid (GLA)*	**2.80 ± 0.017**	**2.45 ± 0.0032**	**2.1 ± 0.11**	**2.62 ± 0.08**
C18:3-9c,12c,15c α-Linolenic Acid (ALA)	0.52 ± 0.08	0.073 ± 0.001	0.036 ± 0.005	0.57 ± 0.21
C18:3(n-3) Trans-Linolenic Acid	ND	-	-	-
C20:0 Arachidic Acid (eicosanoic acid)*	0.80 ± 0.021	0.87 ± 0.00058	0.76 ± 0.041	0.73 ± 0.06
C20:1 Gondoic Acid	0.34 ± 0.01	0.43 ± 0.0027	0.22 ± 0.027	0.42 ± 0.10
C20:1-11c *cis*-Eicosenoic Acid	-	-	-	-
C20:2-11c,14c Eicosadienoic Acid	0.64 ± 0.015	0.42 ± 0.0021	0.44 ± 0.019	0.63 ± 0.15
C20:3-8c,11c,14c Eicosatrienoic (Dihomo-gamma-linolenic) Acid*	**5.29 ± 0.33**	**4.78 ± 0.012**	**3.69 ± 0.054**	**3.27 ± 0.15**
C20:3-11c,14c,17c Eicosatrienoic Acid	0.11 ± 0.006	0.229 ± 0.0	0.027 ± 0.0058	-
C20:4n3 Arachidonic Acid (*cis*-5,8,11,14-eicosatetraenoic acid)*	**45.47 ± 0.59**	**43.92 ± 0.14**	**43.3 ± 1.72**	**40.58 ± 1.91**
C20:5-5c,8c,11c,14c,17c Eicosapentaenoic (EPA) Acid	0.26 ± 0.021	0.1 ± 0.001	0.14 ± 0.059	0.2 ± 0.087

Table 11. Fatty Acid Profile for Hubei Fuxing's ARA-rich oil and ARA-rich Oils that are GRAS

Fatty Acid (%)	Hubei Fuxing ARA-rich oil (n=3)	GRN 730 (n=3)	GRN 326 (n=5)	GRN 94 (n=3)
C21:0 Heneicosanoic Acid	0.063 ± 0.006	0.066 ± 0.0023	0.09 ± 0.0055	-
C22:0 Behenic Acid (docosanoic acid)*	**2.91 ± 0.01**	**3.41 ± 0.0078**	**3.11 ± 0.087**	**2.45 ± 0.35**
C22:1-13c Erucic Acid (*cis*-13-docosenoic acid)	0.07 ± 0.00	0.114 ± 0.0	0.17 ± 0.011	0.15 ± 0.071
C22:1-9c	-	-	-	ND
C22:1-7c	-	-	-	ND
C22:2-13c,16c Docosadienoic Acid	0.12 ± 0.16	ND	0.018 ± 0.0084	-
C22:3-13c,16c,19c Docosatrienoic Acid	-	ND	-	-
C22:4-7c,10c,13c,16c Docosatetraenoic Acid	-	-	-	0.52 ± 0.10
C22:5-4c,7c,10c,13c,16 Docosapentaenoic Acid	-	ND	-	ND
C22:5-7c,10c,13c,16c,19c Docosapentaenoic Acid (DPA)	-	-	-	-
C22:6 -4c,7c,10c,13c,16c,19c Docosaexaenoic Acid (DHA)	0.17 ± 0.18	ND	0.038 ± 0.045	-
C23:0 Tricosanoic Acid	0.057 ± 0.006	ND	-	-
C24:0 Lignoceric Acid*	**9.39 ± 0.27**	**11.29 ± 0.13**	**10.12 ± 0.36**	**6.4 ± 1.72**
C24:1-15c Nervonic Acid	0.30 ± 0.006	0.37 ± 0.00058	0.49 ± 0.049	0.33 ±0.035
Total Fats				
Total Fat	99.75 ± 0.51	95.62 ± 0.16	-	-
Saturated Fat	30.20 ± 0.17	30.23 ± 0.13	27.5±	32.17 ± 0.40
Unsaturated Fat	69.87 ± 0.14	65.304	63.38±	-
Monounsaturated Fat	6.54 ± 0.19	7.40 ± 0.033	-	7.8 ± 0.18
Polyunsaturated Fat	63.27 ± 0.15	57.99 ± 0.18	-	59.68
Trans Fat	0.31 ± 0.021	-	-	-
ω-3 Fat	1.00 ± 0.2	0.40 ± 0.0029	-	-
ω-6 Fat	62.23 ± 0.15	57.59 ± 0.18	-	-
ω-9 Fat	6.28 ± 0.18	-	-	-
Not identifiable fatty acids/others	-	-	-	-

N.D.: not detected, limit of quantitation = 0.01 g/100 g
-: fatty acid residue not quantified
*Fatty acid residues listed as identity criteria in Food Chemicals Codex monograph for ARA from fungal (Mortierella alpina) oil, 13th Edition
Most abundant fatty acid residues indicated in **bold** text: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), eicosatrienoic (C20:3), arachidonic (C20:4), behenic (C22:0), and lignoceric (C24:0) acids

4. Sterol Profiles Among ARA-rich oils From *M. alpina* that are GRAS

Sterols are natural components of plants and animals, many of which are consumed by humans as a part of commonly consumed foods. Desmosterol, a sterol present in human milk (van Brakel et al. 2022) and the primary sterol known to be produced by *M. alpina* (Shimizu et al. 1992; Nes and Nichols 2006), is present in all ARA-rich oils derived from *M. alpina* that are GRAS and received "no questions" letters from the FDA. It is important to note that there is no validated method for the quantification of sterols in ARA-rich oils derived from *M. alpina* and different methodologies have been used to quantify the sterol content in the *M. alpina*-derived ARA-rich oils that are GRAS. Accordingly, an unambiguous quantitative comparison of the sterol profiles of ARA-rich oils that are GRAS is not feasible.

The most abundant sterol residue in Hubei Fuxing ARA-rich oil is 24-methyl desmosterol, which is known to be produced by *M. alpina* through the addition of a methyl group on C24 of desmosterol (Shimizu et al. 1992; Nes and Nichols 2006). The second most abundant sterol is desmosterol. Together, desmosterol and 24-methyl desmosterol account for approximately 75-85% of the sterols present in Hubei Fuxing ARA-rich oil (Table 6), which is consistent with the amount of desmosterol in the other ARA-rich oils derived from *M. alpina* that are GRAS, as well as the initial findings of Shimizu et al. (1992) and Nes and Nichols (2006). Importantly, all the sterols present in the subject of this GRAS Notice are ubiquitous in food, are found in the commonly used sources of fatty acids in infant formula (e.g. corn, palm, safflower, soybean, and sunflower oils), and do not pose safety concerns (Xu et al., 2018; Yang et al., 2019). Because this oil is intended to be substitutional for other ARA-rich oils that are GRAS, infant formulas supplemented with the subject of this GRAS Notice will result in an exposure to sterols that will be similar to those from the subjects of GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2008), and GRN 80/41 (2001). Furthermore, the unsaponifiable content specification of not more than 3% for the subject of this Notice is consistent with the specifications of other ARA-rich oils that are GRAS for use in preterm and infant formula and, together with appropriate production process controls, ensures that the sterol content of the subject of this Notice is controlled in the finished product.

B. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

In human milk, ARA is mainly found in the form of triglycerides, although it can also occur in phospholipids, and accounts for approximately 0.77% of fatty acids (Martin et al., 1993). In general, dietary triglycerides undergo enzymatic hydrolysis in the upper intestine to free fatty acids and 2-monoglycerides. These products then are integrated into bile acid micelles for diffusion into the interior of the intestinal epithelial cells for subsequent incorporation into new or reconstituted triglycerides (Kroes et al., 2003). The new or reconstructed triglycerides

enter the lymph in the form of chylomicrons for transport to the blood, which allows distribution and incorporation into plasma lipids, erythrocyte membranes, platelets, and adipose tissue. The chylomicrons are hydrolyzed by lipoprotein lipase during passage through the capillaries of adipose tissue and the liver to release free fatty acids to the tissues for metabolism or cellular uptake, with subsequent re-esterification into triglycerides and phospholipids for storage as energy or as structural components of cell membranes. The metabolism of fatty acids occurs in the mitochondria following their transport across the mitochondrial membrane in the form of acylcarnitine. Fatty acids are metabolized predominantly via beta-oxidation, a process that involves a shortening of the fatty acid carbon chain and the production of acetic acid and acetyl CoA, which combines with oxaloacetic acid and enters the citric acid cycle for energy production. The degree of transport of fatty acids across the mitochondrial membrane is contingent upon the length of the carbon chain; fatty acids of 20 carbons or more are transported into the mitochondria to a lesser degree than shorter chain fatty acids.

Fatty acids can only be desaturated endogenously up to the n-9 position due to lack of certain enzymes in humans (Kremmyda et al., 2011). For this reason, linoleic (18:2n-6) and linolenic (18:3n-3) acids must be obtained from the diet. Further elongation and desaturation of these fatty acids to produce long-chain PUFA is possible but is not very efficient in humans. Examples of PUFAs include ARA (20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and DHA (22:6n-3). Thus, these fatty acids may be conditionally essential depending on essential fatty acid availability.

During the last trimester of pregnancy, the placenta provides the fetus with ARA. Pre-term birth, which curtails the maternal supply of ARA to the fetus, is associated with sub-optimal neural and visual development, which can be improved by providing exogenous ARA (Kremmyda et al., 2011). After delivery, the premature infant becomes dependent on external sources for its nutritional requirements, due to the shorter period and lesser extent of intrauterine long chain PUFA accumulation. In addition, the infant may have a limited ability to convert essential precursor fatty acids linoleic acid (18:2n-6) to ARA due to reduced concentrations and activity of desaturase enzymes (Martin et al., 2011). Supplementation of these precursor fatty acids may not provide normal concentrations of the downstream fatty acid. Thus, pre-term infants have higher post-natal long chain PUFA requirements than full-term infants, although ARA supplementation can benefit both term and pre-term infants.

C. TOXICOLOGY STUDIES

The safety of *M. alpina*-derived, ARA-rich oils is supported by multiple published and unpublished studies that show that *M. alpina*-derived ARA-rich oils are not genotoxic, not toxic in rats, and well-tolerated in neonatal piglets. These studies have been discussed in depth in previous GRAS notices that received “no questions” and are briefly summarized below. Each ARA-rich oil used in the studies described below is compositionally similar to Hubei Fuxing’s ARA-rich oil.

The lack of genotoxic potential of ARA-rich oil is further supported by an unpublished bacterial reverse mutation assay performed on Hubei Fuxing's ARA-rich oil.

1. Genotoxicity and Toxicology Studies

The previously described published and unpublished genotoxicity studies performed using ARA-rich oils derived from *M. alpina* include reverse mutation assays, chromosomal aberration assays, and micronucleus formation assays. ARA-rich oil was not mutagenic, clastogenic, or aneugenic in these studies (Table 12, the GRAS notice to first describe the study is indicated). ARA-rich oil was used as the test article in published and unpublished acute toxicology studies in rats and mice described in previous GRAS notices. The 50% lethal dose (LD_{50}) reported in these studies was estimated to be greater than the highest dose tested in each study.

The safety of the ARA-rich oil manufactured by Hubei Fuxing is further supported by the following published studies in rats: two 28-day repeat dose toxicology studies (Hempenius et al., 1997; Lewis et al., 2016), a 90-day subchronic toxicology study (Lewis et al., 2016), four 90-day subchronic toxicology studies with an *in utero* exposure (Hempenius et al., 2000; Lina et al., 2006; Casterton et al., 2009; Gao et al., 2014), a reproductive and developmental toxicology study (Falk et al., 2017), and a 16-day tolerance study in neonatal piglets (Merritt et al., 2003). Collectively, the published and unpublished subchronic toxicology studies conducted with the ARA-rich oils derived from *M. alpina* identified no observed adverse effect levels (NOAEL) ranging from 600 to 5,000 mg/kg bw/day. These NOAELS were the highest doses tested with the exception of the studies that reported fatty changes in the liver due to the amount of lipids the rats consumed in these studies. The unpublished toxicology studies that were discussed in previous GRAS notices are summarized in Table 12 and corroborate the findings of the published studies.

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Genotoxicity Studies				
Unpublished study: Suntory, 1991b (GRN 94: page 84, stamped page 96)	SUNTGA25 (25% ARA) from *M. alpina* IS-4	Suntory	Reverse mutation (Ames) assay +/- S9 metabolic activation • Maximum concentration: 5 mg SUNTGA25/plate	5 mg SUNTGA25 was not mutagenic as assessed by the Ames assay regardless of S9 metabolic activation
Hempenius et al (1997) (GRN 41: page 48, Table 13)	ARA-rich oil (38.6% ARA) from *M. alpina*	Gist-brocades	Reverse mutation (Ames) assay+/- S9 metabolic activation (OECD 471) • Maximum concentration: 5000 µg ARA-rich oil/plate Gene mutation test at the TK-locus of mouse lymphoma L5178Y cells +/- S9 metabolic activation (OECD 476) • Maximum concentration: 5 mg ARA-rich oil/mL Mouse micronucleus test in CD-1 mice (OECD 474) • Vehicle control (maize oil) (n=15/sex) • Positive control (0.75 mg mitomycin C/kg) (n=5 males) • 1250 mg ARA-rich oil/kg (n=15 males) • 2500 mg ARA-rich oil/kg (n=15 males) • 5000 mg ARA-rich oil/kg (n=15/sex) In vitro chromosomal aberration test +/- S9 metabolic activation (OECD 473) • Maximum concentration: 5 mg ARA-rich oil/mL	• 5000 µg ARA-rich oil/plate was not mutagenic under the conditions of the Ames assay • 5 mg ARA-rich oil/mL was not mutagenic at the TK-locus of mouse lymphoma L5178Y cells • 5000 mg ARA-rich oil/kg did not produce micronuclei in polychromatic erythrocytes in the mouse micronucleus test • 5 mg ARA-rich oil/mL was not clastogenic under the conditions of the in vitro chromosomal aberration test
Unpublished study: Suntory, 1998b (GRN 94: page 84, stamped page 96)	SUNTGA40S (40% ARA) from *M. alpina* IS-4	Suntory	Reverse mutation assay +/- S9 metabolic activation • Maximum concentration: 5 mg SUNTGA40S	5 mg SUNTGA40S was not mutagenic as assessed by the Ames assay regardless of S9 metabolic activation
Unpublished study: Suntory, 1998c (GRN 94: page 84, stamped page 96)	SUNTGA40S (40% ARA) from *M. alpina* IS-4	Suntory	In vitro chromosomal aberration assay in Chinese hamster fibroblast cell line, +/- S9 metabolic activation • Maximum concentration: 1 mg SUNTGA40S/mL	• Incidence of cells with either structural or numerical aberrations was <5% • 1 mg SUNTGA40S/mL did not produce chromosomal aberrations under the conditions of the assay

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Arterburn et al (2000) (GRN 41: page 48, Table 13 and Appendix 5, page stamped 000235-000236; Appendix 5 page stamped 000241-000242)	ARASCO (48.5% ARA) produced by *M. alpina* ATCC 32222	Martek	Reverse mutation (Ames) assay+/- S9 metabolic activation • Maximum concentration: 5000 µg ARASCO/plate Gene mutation test at the TK-locus of mouse lymphoma L5178Y cells +/- S9 metabolic activation • Maximum concentration: 5000 µg ARASCO/mL In vitro chromosomal aberration test +/- S9 metabolic activation • Maximum concentration: 5010 µg ARASCO/mL	• 5000 µg ARASCO/plate was not mutagenic under the conditions of the Ames assay • 5000 µg ARASCO/mL was not mutagenic at the TK-locus of mouse lymphoma L5178Y cells • 5010 µg ARASCO/mL was not clastogenic under the conditions of the in vitro chromosomal aberration test
Casterton et al (2009) (GRN 326: page 122-128, stamped pages 132-138)	Refined ARA-rich oil (RAO, 43.31% ARA) from *M. alpina*	Cargill	Reverse mutation (Ames) assay+/- S9 metabolic activation (OECD 471) • Maximum concentration: 5000 µg RAO/plate In vitro chromosomal aberration test +/- S9 metabolic activation (OECD 473) • Maximum concentration: 5000 µg RAO/mL Gene mutation test at the TK-locus of mouse lymphoma L5178Y cells +/- S9 metabolic activation (OECD 476) • Maximum concentration: 5000 µg RAO/mL	• 5000 µg RAO/plate was not mutagenic under the conditions of the Ames assay • 5000 µg RAO/mL was not mutagenic at the TK-locus of mouse lymphoma L5178Y cells • 5000 µg RAO/mL was not clastogenic under the conditions of the in vitro chromosomal aberration test

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Lewis et al (2016) (GRN 730: page 30-31, Table 19)	ARA-rich oil (40.34% ARA) from *M. alpina*	Runke Bioengineering	Reverse mutation (Ames) assay+/- S9 metabolic activation (OECD 471) • Maximum concentration: 5 mg ARA-rich oil/plate In vitro chromosomal aberration test +/- S9 metabolic activation • Maximum concentration: 5 mg ARA-rich oil/mL Mammalian erythrocyte micronucleus test in bone marrow of male and female Wistar rats (n=5/sex/group) • Vehicle control (corn oil, 4 mL/kg/day) • 1,000 mg ARA-rich oil/kg/day • 2,500 mg ARA-rich oil/kg/day • 5,000 mg ARA-rich oil/kg/day	• 5 mg ARA-rich oil/plate was not mutagenic under the conditions of the Ames assay • 5 mg ARA-rich oil/mL was not clastogenic under the conditions of the in vitro chromosomal aberration test • 5000 mg ARA-rich oil/kg did not produce micronuclei in the mammalian micronucleus test
Acute Toxicity				
Unpublished report: Suntory, 1991a (GRN 94: page 83, stamped page 95)	SUNTGA25 (25% ARA) from *M. alpina* IS-4	Suntory	Acute toxicity study in male ICR (SPF) mice (n=5/group): • Olive oil vehicle control • 2,000 mg SUNTGA25/kg	LD_{50} > 2,000 mg SUNTGA25/kg
Unpublished study: Glaza, 1992 (GRN 41: page 45, Table 9, and Appendix 5, pages stamped 000268-270)	ARASCO (38-44% ARA) strain not provided	Martek	Acute oral toxicity in Crl:CD BR albino rats (n=5/sex) • 20,000 mg ARASCO/kg	LD_{50} > 20,000 mg ARASCO/kg
Boswell et al. (1996) (GRN 41: page 45, Table 9)	ARASCO (48.5% ARA) produced by *M. alpina*	Martek	Acute oral toxicity study in male and female Crl: CD BR albino rats (n=10 rats/sex) • 20 g ARASCO/kg	LD_{50} > 20,000 mg/kg
Unpublished report: Glaza, 1997 (GRN 41: page 45, stamped page 145, Table 9)	Microencapsulated ARASCO/DHASCO blend (ARA concentration not described)	Martek	Acute oral toxicity in rats: • 5 g microencapsulated ARASCO/DHASCO blend/kg	LD_{50} > 5 g microencapsulated ARASCO/DHASCO blend/kg
Hempenius et al. (1997) (GRN 41: page 45, Table 9)	ARA-rich oil (38.6% ARA) from *M. alpina*	Gist-brocades	Acute oral toxicity study in Wistar rats (n=5/sex) (OECD 401): • Maize oil vehicle control (20 mL/kg) • 18,200 mg ARA-rich oil/kg (6,200 mg ARA/kg)	LD_{50} > 18,200 mg ARA-rich oil/kg, or 6,200 mg ARA/kg

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Unpublished report: Suntory, 1998a (GRN 94: page 83, stamped page 95)	SUNTGA40S (40% ARA) from *M. alpina* IS-4	Suntory	Acute toxicity study in male ICR (SPF) mice (n=5/group): • Olive oil vehicle control • 2,000 mg SUNTGA40S/kg	LD_{50} > 2,000 mg SUNTGA40S/kg
Lewis et al (2016) (GRN 730: page 30-31, Table 19)	ARA-rich oil (40.34% ARA) from *M. alpina*	Runke Bioengineering	Acute toxicity study in female Wistar rats (n=10/group) • 5,000 mg ARA-rich oil/kg	LD_{50} > 5,000 mg ARA-rich oil/kg
Unpublished report: Gao, 2017 (GRN 730: page 31, Table 20)	ARA-rich oil (42.1% ARA) from *M. alpina* LU 166	Linyi Youkang Biology	Acute oral toxicity study in rats (n=5/sex/group): • Water control • Sunflower oil vehicle control • 15,200 mg ARA-rich oil/kg	LD_{50} > 15,200 mg ARA-rich oil/kg
Repeated Oral Toxicity and Subchronic Toxicity				
Unpublished study: Suntory, 1992a (GRN 94: Page 85, stamped page 97, Table VI-4)	SUNTGA25 (25% ARA) from *M. alpina* IS-4	Suntory	14-day oral toxicity range finder study in rats: • Standard diet • 2.5% SUNTGA25 in the diet (709 mg ARA/kg) • 5% SUNTGA25 in the diet (1,320 mg ARA/kg) • 10% SUNTGA25 in the diet (2,961 mg ARA/kg)	No toxicity reported at the highest level of SUNTGA25 in the diet, or 2,961 mg ARA/kg
Unpublished report: Suntory, 1992b (GRN 94: page 86-88, stamped pages 98-100, Table VI-5)	SUNTGA25 (25% ARA) from *M. alpina* IS-4	Suntory	A 90-day oral toxicology study in rats (n=10/sex/group): • Note: test group diets were not standardized for lipid content • Standard diet: 0% SUNTGA25, 4.4% fat total fat in diet • Standard diet + 0.8% SUNTGA25 (150 mg ARA/kg/day), 5.2% total fat in diet • Standard diet + 2.0% SUNTGA25 (300 mg ARA/kg/day), 6.4% total fat in diet • Standard diet + 5.0% SUNTGA25 (750 mg ARA/kg/day), 9.4% total fat in diet	NOAEL: 0.8% SUNTGA25, 150 mg ARA/kg/day NOAEL was set based on statistically significant increases in the liver weights in the standard diet + 2.0% ARA-rich oil- and the standard diet + 5.0% ARA-rich oil- treated groups

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Boswell et al. (1996) (GRN 41: page 45, Table 9) (GRN 94, stamped page 356, Table 1	ARASCO (48.5% ARA) produced by *M. alpina*	Martek	28 day repeated oral toxicity study in male and female Crl: CD (SD)BR VAF/Plus rats (all diets had the same fat content) • Vehicle control: 3750 mg high oleic sunflower oil/kg/day (n=10 rats/sex) • 50 mg ARASCO/kg/day (n=5 rats/sex) • 1,000 mg ARASCO/kg/day (n=5 rats/sex) • 2,500 mg ARASCO/kg/day (n=10 rats/sex)	No toxicity observed in rats receiving 2,500 mg ARASCO/kg/day
Hempenius et al. (1997) (GRN 41: page 45-46, Table 10)	ARA-rich oil (32.7% ARA) from *M. alpina*	Gist-brocades	28-day repeated oral toxicity study in Wistar rats (n=5/sex/group) (OECD 407): • Note: All animals received the same dose volume of 5 mL oil/kg/day • Corn oil vehicle control, 5 mL/kg/day (n=10/sex/group) • 100 mg ARA-rich oil/kg/day • 600 mg ARA-rich oil/kg/day • 2,000 mg ARA-rich oil/kg/day • 3,000 mg ARA-rich oil/kg/day	No toxicity observed at 3,000 mg ARA-rich oil/kg/day corresponding to 1000 mg ARA/kg/day
Koskelo et al. (1997) (GRN 41, Pages 46-47, stamped pages 000146- 000147, Table 11)	ARASCO (51.4% ARA) produced by *M. alpina*	Martek	A 90-day oral toxicity study in Sprague Dawley rats (n=20/sex/group): • Untreated • Note: all animals in the vehicle control and test groups received 3 mL oil/kg/day to control for fat content • Vehicle control (high-oleic sunflower oil) • 1,000 mg ARA-rich oil/kg/day diluted with high-oleic sunflower oil • 2,500 mg ARA-rich oil/kg/day diluted with high-oleic sunflower oil	NOAEL: 2,500 mg ARA-rich oil/kg/day, corresponding to ~1.0 g ARA/kg/day 1 male from high-dose ARASCO group died due to intubation error and 1 female from high-dose ARASCO group found dead during the study. Deaths not attributed to administration of ARASCO
Unpublished study: Lina, 1997 (GRN 41: page 47, stamped page 000147, Table 11)	ARASCO (ARA concentration not described)	Gist-brocades	A 90-day *in utero* phase, oral toxicity study in rats • 4,900 mg/kg bw/day (highest dose) of ARASCO • Other doses or vehicle not described in publicly available summary	NOAEL: 4,900 mg ARASCO/kg bw/day

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Unpublished study: Suntory, 1997 (GRN 94: page 89-106, stamped page 101-118, Tables VI-6, VI-7,VI-8,VI-9,VI-10, VI-11, V1-12, VI-13; Figures VI-1, VI-2)	SUNTGA40S (39.7% ARA) from *M. alpina* IS-4	Suntory	A 13-week subchronic oral toxicology study in Crj:CD (SD strain) rats: • Standard diet • Standard diet + 2% soybean oil • Standard diet + 1% DHA and ARA rich oil mean intakes of 591 mg ARA-rich oil/kg/day in males and 696 mg ARA-rich oil/kg/day in females • Low dose, ARA-rich oil mean intakes of 283 mg ARA-rich oil/kg/day in males and 341 mg ARA-rich oil/kg/day in females • Middle dose, mean intakes of 578 mg ARA-rich oil/kg/day in males and 674 mg ARA-rich oil/kg/day in females • High dose, mean intakes of 1158 mg ARA-rich oil/kg/day in males and 1353 mg ARA-rich oil/kg/day in females	NOAEL: High dose, mean intakes of 1158 mg ARA-rich oil/kg/day in males and 1353 mg ARA-rich oil/kg/day in females

Hempenius et al. (2000) (GRN 94: Attachment 1, Table 4, page stamped 000359)	ARA-rich oil (38% ARA)	Gist	13 week subchronic oral toxicology study in rats with an *in utero* phase (OECD 408):
			• Standard diet
			• All diets below were the standard diet with the same levels of total fat:
			  • Vehicle control: corn oil (130,000 ppm)
			  • 3,000 ppm ARA-rich oil
			  • 15,000 ppm ARA-rich oil
			  • 75,000 ppm ARA-rich oil
			  • 75,000 ppm ARA-rich oil + 55,000 ppm DHA-rich oil
				• NOAEL: 15,000 ppm ARA-rich oil (approximately 970 mg ARA-rich oil/kg/day)
				• Evidence of lipid toxicity was observed in rats receiving 75,000 ppm ARA-rich oil: increased incidence of hepatocellular vacuolation (females), oil droplets in mesenteric lymph nodes and intestinal villi (males and females)

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Lina et al. (2006) (GRN 326 page 152-153, stamped pages 162-163, and Section 8.2)	SUNTGA40S (41.5% ARA) from *M. alpina* IS-4	Suntory	90-day subchronic oral toxicity study in Wistar outbred (Crl:(WI)WU BR) rats with a 4-week *in utero* phase (OECD 408): • Standard diet • All diets below were the standard diet with the same amount of added fat (5.76%): o Vehicle control: 5.76% corn oil o 0.5% ARA-rich oil in the diet o 1.5% ARA-rich oil in the diet o 5.0% ARA-rich oil in the diet o 3.65% ARA-rich oil and 2.11 % DHA-rich tuna oil in the diet	NOAEL: 5% ARA-rich oil in the diet, approximately 3,000 mg ARA-rich oil/kg/day in F_0 and F_1 rats
Casterton et al. (2009) GRN 326: page 135-152	Refined ARA-rich oil (RAO, 43.31% ARA) from *M. alpina*	Cargill	13 week subchronic oral toxicology study in Wistar rats with an *in utero* phase (OECD 408): • Standard diet • All diets below were the standard diet with the same amount of total fat (5% added fat): • Corn oil (high-fat control group) • 0.5% RAO • 1.5% RAO • 5.0% RAO	NOAEL: 5% RAO in the diet, approximately 3,170 mg RAO/kg/day
Gao et al. (2014) (GRAS 730: page 32, Table 20)	ARA-rich oil (48.3% ARA) from *M. alpina* XM027	Xiamen Kingdomway Group Company and Inner Mongolia Kingdomway Pharmaceutical Limited	A 90-day subchronic oral toxicity study in Sprague Dawley rats of F1 after 4-week *in utero* phase of F_0 (n=10/sex/group): • Standard diet • High fat control: 5% weight/weight fish oil in the diet • 0.5% ARA-rich oil in the diet • 1.5% ARA-rich oil in the diet • 5.0% ARA-rich oil in the diet	NOAEL: 5% ARA-rich oil in the diet, approximately 3,750 mg ARA-rich oil/kg/day in F_0 females, 2,850 mg ARA-rich oil/kg/day in F_0 males, 4,850 mg ARA-rich oil/kg/day in F_1 females, and 4,480 mg ARA-rich oil/kg/day in F_1 males

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Lewis et al. (2016) GRN 730: page 32 and Table 20	ARA-rich oil (40.34% ARA) from *M. alpina*	Runke Bioengineering Co	28-day repeated oral toxicity study in male and female Wistar rats, all dose volumes were 4 mL/kg (n=10/sex/group): • Water • Vehicle control (corn oil) • 1,000 mg ARA-rich oil/kg/day • 2,500 mg ARA-rich oil/kg/day • 5,000 mg ARA-rich oil/kg/day 90-day repeated oral toxicity study in male and female Wistar rats, all dose volumes were 4 mL/kg (n=20/sex/group): • Water • Vehicle control (corn oil) • 1,000 mg ARA-rich oil/kg/day • 2,500 mg ARA-rich oil/kg/day • 5,000 mg ARA-rich oil/kg/day • Recovery group -Vehicle control (corn oil) • Recovery group - 5,000 mg ARA-rich oil/kg/day	• No toxicity observed in the highest dose tested in the 28-day toxicology study: 5,000 mg ARA-rich oil/kg/day • NOAEL: 5,000 mg/kg/day
Developmental/Reproductive Toxicity				
Unpublished study: Henwood, 1995 (GRN 41: pages stamped 000291-00292)	ARASCO (38-44% ARA)	Martek	Developmental oral toxicity study in mated female Sprague-Dawley (Crl: CD (SD)BR VAF/Plus rats (n=25/group) from gestation days 6 through 20: • Standard diet • Vehicle control: 2,500 mg high-oleic sunflower oil/kg/day • 1,000 mg ARASCO/kg/day • 2,500 mg ARASCO/kg/day	No test article-related effects were noted in any treatment group

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Falk et al. (2017) (GRN 730: page 32, Table 20)	ARA-rich oil (40.3% ARA) from *M. alpina*	Runke Bioengineering	Prenatal developmental toxicity study in pregnant female Wistar rats (n=24/group): • Untreated control • Vehicle control: corn oil • 1,000 mg ARA-rich oil/kg/day • 2,500 mg ARA-rich oil/kg/day • 5,000 mg ARA-rich oil/kg/day Reproductive toxicity study in male and female Wistar rats (n=20 males and 24 females/group) • Vehicle control: corn oil • 1,000 mg DHA-rich oil/kg/day • 2,500 mg DHA-rich oil/kg/day • 5,000 mg DHA-rich oil/kg/day	• No observed maternal or embryofetal toxicity at 5,000 mg ARA-rich oil/kg/day • No observed paternal or maternal treatment related reproductive toxicity at 5,000 mg ARA-rich oil/kg/day
Neonatal Piglet Studies				

Mathews et al. (2002) (GRN 80: page 13, stamped page 000019)	ARASCO (ARA concentration not described)	Martek	16-day neonatal piglet tolerance study (n=10/group):
			• Control 34.2 g essential fatty acids/100 g fat
			• Control: 0 g essential fatty acid/100 g fat
			• All diets below contained 34.2 g essential fatty acids/100 g fat + ARA and DHA from different sources:
			  ○ 0.6 g ARA/100 g fat and 0.3 g DHA/100 g fat from egg phospholipid
			  ○ 0.6 g ARA/100 g fat and 0.3 g DHA/100 g fat from ARASCO and DHASCO
			  ○ 0.6 g ARA/100 g fat and 0.3 g DHA/100 g fat from ARASCO and DHASCO + choline, cholesterol and soy lecithin
				No test article-related effects were noted in any of the treatment groups

Table 12. Toxicology Studies that Support the Safe Use of ARA-rich oils from *M. alpina*

Reference (GRAS Notice)	Test Substance	Manufacturer	Study Type	Conclusions
Merritt et al (2003) (GRN 94, page 11-13, page 119-170, stamped pages 131-182) (GRN 326: Page 151, stamped page 000161)	SUNTGA40S (40% ARA) from *M. alpina* IS-4	Suntory	A 16-day tolerance study in piglets (n=6/sex/group) • Control Formula • Control Formula + 5X ARA (2.4 mg ARA-rich oil/g formula) • Control Formula + 5X DHA (2.7 mg DHA-rich oil/g formula) • Control Formula 3X ARA w/3X DHA (1.5 mg ARA-rich oil + 1.6 mg DHA-rich oil/g formula)	No test article-related effects were noted in any of the treatment groups

2. Unpublished Hubei Fuxing ARA-rich Oil Genotoxicity Study

The mutagenicity of Hubei Fuxing's ARA-rich oil was assessed in an unpublished Ames assay using the plate incorporation method. This assay was performed using the following bacterial strains of *S. typhimurium* histidine auxotroph: TA97, TA98, TA100, and TA102. The Ames test was also performed in the presence (+S9) or absence (-S9) of PCB-induced rat liver homogenate as an in vitro activation system. ARA-rich oil was used at 0.2, 0.5, 1, 2.5, and 5 mg/dish in 0.1 mL for each strain of *S. typhimurium.* The ARA-rich oil was diluted in dimethyl sulfoxide (DMSO), which was used as the vehicle control. The spontaneous revertants were included as the indicated strains of *S. typhimurium* with no treatment as the negative control. A positive control for all strains, including the S9-treated strains, was also included in the assay. The positive control for strains TA97, TA98, and TA102 in the absence of S9 was 50 µg/plate Dexon (polyglycolic acid). The positive control for TA100 in the absence of S9 was 2.5 µg/plate sodium azide. In the presence of S9 enzymatic activation, TA97, TA98, and TA100 used 10 µg/plate 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (2-AF) as a positive control. The positive control for TA102 with S9 activation was 50 µg/plate 1,8-dihydroxy anthraquinone. The assay was repeated twice with the average number of colonies shown with standard deviations below in Table 13.

If the ARA-rich oil treatment groups had twice the number of colonies as the spontaneous revertant group, then ARA-rich oil would be considered genotoxic. None of the treatments, in the presence or absence of S9 activation, caused an increase in colony number over the spontaneous revertant control. No dose response relationship was observed in any of the strains of *S. typhimurium*. The positive controls for each strain had a robust increase in colony number. These results indicate that ARA-rich oil is not genotoxic under the conditions used in this assay.

The lack of genotoxic potential of Hubei Fuxing's ARA-rich oil is also supported by an Ames assay, in vitro chromosomal aberration test, and an *in vivo* mammalian erythrocyte micronucleus test performed on the ARA-rich oil in GRN 730, another ARA-rich oil generated from *M. alpina*. The ARA-rich oil described in GRN 730 yielded negative results for these genotoxicity studies (Lewis et al., 2016).

Table 13. Ames Test Results with ARA-Rich Oil

S. typhimurium Strain		TA97		TA98		TA100		TA102	
	mg/plate	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
ARA-rich oil	0.2	150.5 ± 0.71	158.5 ± 0.71	42.5 ± 0.71	42.5 ± 2.12	159.5 ± 0.71	164.5 ± 0.71	247.0 ± 1.41	237.0 ± 1.41
	0.5	153.0 ± 1.41	157.0 ± 4.24	42.0 ± 1.41	42.0 ± 1.41	160.0 ± 2.83	157.5 ± 13.44	248.0 ± 4.24	239.5 ± 4.95
	1	150.5 ± 3.54	159.5 ± 2.12	43.0 ± 1.41	41.0 ± 1.41	166.0 ± 2.83	166.0 ± 2.83	246.0 ± 5.66	249.0 ± 4.24
	2.5	154.5 ± 0.71	161.0 ± 2.83	42.0 ± 1.41	44.0 ± 1.41	162.5 ± 0.71	166.0 ± 5.66	248.5 ± 10.61	258 ± 2.83
	5	158.5 ± 0.71	156.0 ± 9.90	46.5 ± 0.71	45.5 ± 0.71	161.5 ± 2.12	158.5 ± 2.12	254.5 ± 3.54	253.5 ± 9.19
DMSO		154.0 ± 4.24	153.0 ± 2.83	41.0 ± 1.41	41.5 ± 0.71	156.5 ± 3.54	156.0 ± 1.41	256.5 ± 0.71	258.5 ± 2.12
Spontaneous revertant		150.0 ± 2.83	156.0 ± 2.83	42.5 ± 0.71	42.0 ± 2.83	153.0 ± 2.83	157.5 ± 0.71	256.5 ± 7.78	261.5 ± 0.71
Positive Control Group (ug/plate)									
NaN_3	2.5	-	-	-	-	1611.5 ± 40.31	-	-	-
2-AF	10	-	1158.5 ± 58.69	-	1845.0 ± 25.46	-	1907.0 ± 0.00	-	-
Dexon (polyglycolic acid)	50	2428.0 ± 63.64	-	2357.0 ± 14.14	-	-	-	1195.0 ± 63.64	-
1,8-dihydroxy anthraquinone	50	-	-	-	-	-	-	-	1535.0 ± 7.07

Average of two experiments shown with standard deviation
2-AF: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide
DMSO: dimethyl sulfoxide, served as vehicle control

D. CLINICAL STUDIES

The clinical studies discussed in previous GRNs are briefly summarized below and in Table 14. Studies using 0.64-0.72% of total fatty acids as ARA (0.72% - Birch et al., 2005, 2007; 0.64% - Birch et al., 2010) demonstrated the safety of ARA-rich oil derived from *M. alpina* in term infants. No studies reported adverse effects attributed to the ingestion of ARA or ARA-rich oil. There have been no adverse effects reported on the consumption of ARA-rich oils in pre-term infants when used at levels up to 0.91% total fatty acid content (Almaas et al., 2015, 2016; Carnielli et al., 2007; Clandinin et al., 2005; Groh-Wargo et al., 2005; Henricksen 2008). These studies used fungal sources of ARA-rich oil. We incorporate by reference the clinical studies described in GRN 326 (pages 61-88), GRN 730 (pages 35-44), and GRN 963 (pages 26-29).

Some of the published studies summarized in previous GRAS Notices are subgroup analyses of earlier published studies. For example, in the initial published report of the DIAMOND study, Birch et al. 2010, described the safety endpoints associated with the study, while Colombo et al. (2011), Drover et al. (2011), and Drover et al. (2012) are subgroup analyses of the study and do not describe any new safety endpoints. Because Birch et al., 2010 contains the primary safety data for the DIAMOND study, it describes the relevant safety data from the study and is summarized in Table 14 below. The subgroup analyses associated with the DIAMOND study are noted in the table but are not reviewed because those studies do not describe safety outcomes. Accordingly, any subgroup analyses associated with the primary study publication are indicated in Table 14 with the primary study but were not summarized.

The latest ARA-rich oil GRAS notice that received no questions from the FDA was in 2021. To determine if any additional clinical studies have been published on the safe use of ARA-rich oils for infants, a literature search was performed using PubMed on May 25, 2022. The search terms were “arachidonic acid” AND “infant,” with the results constrained to clinical trials published between 2021 and May 25, 2022. Publications that are not relevant to assessing the safety of ARA in infant formula intended for oral feeding (i.e. those that studied non-infant ARA supplementation or studied ARA as a supplement and not as an ingredient in an infant formula), or did not use fungal-derived ARA were not reviewed. Publications that did not report safety parameters were not reviewed. Only initial published reports of clinical studies, as defined above, were reviewed in Table 14. Any recent publication that was a subgroup analysis of an earlier clinical trial are indicated in Table 14. Finally, only studies that were appropriately controlled, i.e. studies that used a control formula that was not supplemented with ARA and a test formula that was supplemented with fungal-derived ARA, were reviewed.

One primary study that was not reviewed in previous GRAS notices was identified and summarized in Table 14, Salas-Lorenzo et al., (2019). Its subgroup analyses publications are indicated in the table.

These studies demonstrate that fungal-derived ARA-rich oils, providing up 0.91% of total fatty acid content of total fatty acids as ARA, are well-tolerated in pre-term and term infants. Although some of the studies reported adverse events, the adverse events were equally distributed among the control and test groups and the investigators reported that they were unrelated to the study formula. Therefore, the weight of the evidence indicates that there is reasonable certainty that the ARA-rich oil that is the subject of this Notice is safe per the intended uses and use levels.

Table 14. Pre-term and Term Infant Clinical Studies Using Fungal-derived ARA-rich Oil

Reference (GRAS Notice)	Study Design and Population	Treatment Groups	Outcomes and Safety Parameters
Studies in Preterm Infants			
Clandinin et al., 2005 (GRN 326, pages 63 and 109)	A prospective, randomized double-blind study in preterm infants; 92 weeks post-menstrual age (PMA) with follow up in second phase at 118 weeks PMA	• Control: infant formula • Test Group 1: Formula with 34 mg ARA + 17 mg algal DHA/100 kcal • Test Group 2: Formula with 34 mg ARA + 17 mg fish DHA/100 kcal • Reference Group: term infants (n=105) breast-fed for ≥ 4 months (included in the second phase) • The algal DHA-rich oil and fungal ARA-rich oils were provided by Martek Biosciences • The fish DHA-rich oil was provided by Roche Vitamins Inc.	• In the first phase (before 40-week postmenstrual age), 119 subjects were enrolled in the control group, 112 were enrolled in Test Group 1, and 130 were enrolled in Test Group 2 o 56 infants (21 in Control Group, 17 in Test Group 1, 18 in Test Group 2) discontinued. o The most common reasons for discontinuation were formula intolerance (n = 15), medical complications unrelated to the study (n = 13), and parental request (n = 11). o There were no differences among groups in discontinuation rates or distribution of reasons for discontinuation. • In the second phase (40 to 118 weeks postmenstrual age), 83 subjects were enrolled in the Control Group, 72 subjects were enrolled in the Test Group 1, 90 subjects were enrolled in Test Group 2, and 105 were enrolled in Reference Group o 95 infants (21 in Control Group, 20 in Test Group 1, 25 in Test Group 2, and 29 in Reference Group) discontinued in the second phase. o The most common reasons for discontinuation were formula intolerance (n = 15), medical com- plications unrelated to the study (n = 13), and parental request (n = 11). o There were no differences among groups in discontinuation rates or distribution of reasons for discontinuation. • Results showed that the weight of the infant group given ARA together with DHA was significantly ($p<0.05$) greater than the control group from 66 to 118 weeks PMA but did not differ from infants in the reference group at 118 weeks PMA. • Bayley mental (MDI) and psychomotor development (PDI) scores at 118 weeks PMA (18 months after term) were higher in infants given ARA/DHA supplemented formula compared to the control group. The MDI and PDI scores for the infants in the breast-fed term reference group were near the reference norm and significantly higher than the preterm groups. • Mean weight, length, and head circumference and respective growth rates did not differ among the preterm groups. • Analysis of clinical data including the severity of medical conditions relating to prematurity, serum chemistry, and hematology found no safety issues related to the supplemented formulas. There were no increases in morbidity or adverse events in the groups given supplemented formulas relative to the control.

Table 14. Pre-term and Term Infant Clinical Studies Using Fungal-derived ARA-rich Oil

Reference (GRAS Notice)	Study Design and Population	Treatment Groups	Outcomes and Safety Parameters
Groh-Wargo et al., 2005 (GRN 326, page 62)	A controlled, double blind study conducted up to 40-weeks gestational corrected age of infants Sixty preterm infants with birth weights from 750 to 1800 g and gestational age at birth <33 weeks	• Control: milk formula (n=22) • Test Group 1: milk formula supplemented with 0.42% ARA (egg-derived triglyceride) and 0.26% DHA (fish oil); (% based on grams/100 grams total fatty acids) (n=18) • Test Group 2: milk formula supplemented with 0.42% ARA (fungal oil) and 0.26% DHA (fish oil); (% based on grams/100 grams total fatty acids) (n=20) • The manufacturers of the DHA- and ARA-rich oils were not provided.	• One infant who received a diagnosis of a rare neurologic disorder and two infants who were breast-fed exclusively throughout the study were excluded from the analyses. • 16 subjects dropped out of the study o 7 subjects in the Control Group and 1 subject in Test Group 1 withdrew due to switching to a non-study formula per physician recommendation o 2 subjects withdrew from Test Group 1 withdrew voluntarily due to the parent or investigator. o 2 subjects in Test Group 1 and 3 subjects in Test Group 2 withdrew due to noncompliance with study visits o 1 subject in Test Group 2 died due to reasons unrelated to the study participation. • No significant differences were seen among the three groups were seen at any time point in weight, length, or head circumference. • Bone mineral content and bone mineral density did not differ among groups. At 12 months, term corrected age (TCA) infants who were fed ARA/DHA-supplemented formulas had significantly greater lean body mass and significantly less fat mass than infants who were fed the non-supplemented control formula. • The ARA/DHA-supplemented formulas supported normal growth and bone mineralization in premature infants who were born at < 33-week gestation. No differences among the groups were seen in the percentage of infants with adverse clinical complications. • At 12 months TCA, preterm infants that were fed the ARA/DHA supplemented formula had increased lean body mass and significantly less fat mass by one year of age than infants fed non-supplemented formula. • The authors concluded supplementation of infant formula with ARA and DHA had a beneficial effect on growth and lean body mass.

Table 14. Pre-term and Term Infant Clinical Studies Using Fungal-derived ARA-rich Oil

Reference (GRAS Notice)	Study Design and Population	Treatment Groups	Outcomes and Safety Parameters
Carnielli et al., 2007 (GRN 326, page 115 and Table 28 on page 82)	Randomized pre-term infants, from birth to 7 months	• Control Group: non-supplemented formula (n=11) • Test Group: Fungal ARA (0.84%) 12.0 mg + DHA (fish oil), 7.1 mg per 100 mL of formula (n=11) • DHA- and ARA-rich oils were the single cells oils DHASCO and ARASCO provided by Martek Biosciences	• Measured absolute long chain PUFA synthesis and percentage of long chain PUFA synthesis relative to dietary intake and plasma phospholipids. • All infants grew normally during the trial (7 months) and no significant difference between groups was found in weight gain. • The authors did not report the adverse events.
Henricksen et al., 2008 (GRN 326, page 67 and page 118)	Randomized, double-blinded, placebo-controlled intervention trial in very low birth weight infants. Infants were given milk + oil for an average of 63 days from birth to discharge from the hospital	• Control: human milk with placebo (n=48) • Test group: human milk with 0.5 mL oil (containing 31 mg ARA plus 32 mg DHA) per 100 mL milk (n=44)	• During the trial, 6 infants in the control and 6 infants in the test group were excluded. o The control group had 2 infants excluded due to adverse events, 2 due to death. 1 due to congenital abnormalities, and one parent withdrew consent o The test group had 5 infants excluded due to adverse events, and 1 due to enteral feeding. • No significant difference in registered adverse events between the control and the test group. • No effects in growth. • Subsequent subgroup analyses of this study are described in: Westerberg et al., 2011; Almaas et al., 2015; and Almaas et al., 2016.

Table 14. Pre-term and Term Infant Clinical Studies Using Fungal-derived ARA-rich Oil

Reference (GRAS Notice)	Study Design and Population	Treatment Groups	Outcomes and Safety Parameters
Studies in Term Infants			
Birch et al., 2005 (GRN 326, page 64 and page 111)	Doubly masked, randomized controlled trial with 39-week duration and follow up until 52 weeks, following study initiation in term infants.	• Control group: infant formula (n=52) • Test group: infant formula supplemented with 0.72% ARA (fungal oil) and 0.36% DHA (algal oil). Percentages in diet given as % of total fatty acids. (n=51) • ARA-rich and DHA-rich oils were the single cell oils ARASCO and DHASCO provided by Martek Biosciences	• Over the course of the trial, 5 subjects in the control group and 5 subjects in the test group withdrew due to gastrointestinal intolerance; 1 subject in the control group and 2 subjects in the test group withdrew due to illness unrelated to the study formula, and 2 subjects in the control group and 2 subjects in the test group were lost to follow-up. • Evaluation of sweep visual evoked potential (VEP) acuity in the LCPUFA supplemented group was significantly better than that in the non-supplemented control group at all time points measured ($p<0.001$ to 0.01). • Red blood cell concentration of ARA was 15-18% higher in the LCPUFA supplemented group than in the control group. Red blood cell DHA concentrations in the LCPUFA group were 215% higher than in the control group by 39 weeks. Both increases were statistically significant ($p<0.001$ to 0.01). • For both groups, all anthropometric outcome data were normally distributed. No significant effect of diet was found on growth evaluated by weight, length, or head circumference, and both diets were well tolerated.

Table 14. Pre-term and Term Infant Clinical Studies Using Fungal-derived ARA-rich Oil

Reference (GRAS Notice)	Study Design and Population	Treatment Groups	Outcomes and Safety Parameters
Birch et al., 2007 (GRN 326, Page 113)	Randomized trial with follow up study at 4 years of children that had been fed supplemented diets for 17 weeks during infancy	• Control Group: infants fed commercial un-supplemented formula (n=26) • Test Group 1: Infants fed supplemented formula with 0.35% DHA (algal oil) (% based on total fatty acid content.) (n=26) • Test Group 2: Infants fed supplemented formula with 0.72% ARA (fungal oil) and 0.36% DHA (algal oil) (% based on total fatty acid content.) (n=27) • Reference Group: 40 breast-fed infants that had been enrolled in the same 17-week randomized trial • DHA- and ARA-rich oils were the single cells oils DHASCO and ARASCO provided by Martek Biosciences	• 26 subjects were enrolled in the Control Group, 26 subjects were enrolled in Test Group 1, 27 subjects were enrolled in Test group 2, and 40 were enrolled in the Reference Group. • 23 subjects in the Control Group, 22 subjects in Test Group 1, 23 subjects in Test Group 2, and 38 subjects in the Reference Group completed the 4 month-feeding protocol. l • 19 subjects in the Control Group, 16 subjects in Test Group 1, 17 subjects in Test Group 2, and 32 subjects in the Reference Group completed the 4-year follow-up testing. • Most of the loss to follow-up occurred during the initial weeks following enrollment (prior to the 4-month visit) and was due to their pediatricians' recommendations to switch to a soy protein- based formula following symptoms suggestive of lactose or cow milk protein intolerance. • At 4 years of age, the group that had received the control formula as infants had significantly poorer visual activity ($p<0.03$) and verbal IQ ($p<0.003$) than the children who were breast-fed for an average of 43 weeks or those who had been fed formula containing ARA/DHA during the first 17 weeks of life. The formula supplemented group had visual acuity and verbal IQ scores that did not differ significantly from breast-fed children at 4 years of age. The result of this trial suggested that ARA/DHA supplementation of infant formula for at least the first 4 months after birth supports visual activity and IQ maturation similar to that of breast-fed infants. • No discussion of adverse events or safety parameters was reported.

Table 14. Pre-term and Term Infant Clinical Studies Using Fungal-derived ARA-rich Oil

Reference (GRAS Notice)	Study Design and Population	Treatment Groups	Outcomes and Safety Parameters
Birch et al., 2010 (GRN 730, Table 23 on page 42)	Double-masked, randomized trial in healthy term infants, first 12 months of life (from days 1-9), sole source of nutrition until < 4 months of age. This report is from the DIAMOND (DHA Intake And Measurement of Neural Development) study.	• Control Group: non-supplemented infant formula (n=86) • Test group 1: 0.64% ARA + 0.32% DHA supplemented infant formula (n=84) • Test Group 2: 0.64% ARA + 0.64% DHA supplemented infant formula (n=85) • Test Group 3: 0.64% ARA + 0.96% DHA supplemented infant formula (n=88) • The DHA- and ARA-rich oils were derived from *Crypthecodinium cohnii* and *Mortierella alpina,* respectively, and provided by Martek Biosciences.	• 56 subjects (66%) in the Control Group, 64 subjects (77%) in Test Group 1, 59 subjects (70%) in Test Group 2, and 65 subjects (75%) in Test Group 3 completed the study. o 8 subjects were lost to follow up and 21 discontinued the intervention in the Control Group. o 6 subjects were lost to follow up and 13 discontinued the intervention in the Control Group. o 5 subjects were lost to follow up and 20 discontinued the intervention in the Control Group. o 6 subjects were lost to follow up and 16 discontinued the intervention in the Control Group. o The reasons for discontinuation (physician-assessed formula intolerance, parent removed infant, missed visits, reduced formula intake, other) were similar between the formula groups. • Infants fed control formula had significantly poorer visual evoked potential visual acuity at 12 months of age than infants that received any of the ARA + DHA supplemented infant formula. No difference between ARA + DHA groups was observed. • No significant effects on weight, length, bowel movements, or adverse events. • Infant formulas were well-tolerated and all groups had normal growth throughout the first 12 months of life. • Subsequent subgroup analyses of the DIAMOND study were described in Colombo et al., 2011; Drover et al., 2011; Drover et al., 2012.

Table 14. Pre-term and Term Infant Clinical Studies Using Fungal-derived ARA-rich Oil

Reference (GRAS Notice)	Study Design and Population	Treatment Groups	Outcomes and Safety Parameters
Salas Lorenzo et al., 2019	Interventional, randomized and double-blinded study of 176 full term, healthy infants (COGNIS study: a Neurocognitive and Immunological Study of a New Formula for Healthy Infants))	• Reference group: breast fed infants (n=50) • Test group 1: infants fed with standard formula (n=85) • Test group 2: infants fed with experimental formula, with 15.8 mg/100 mL fungal oil ARA and 11.2 mg/100 mL DHA (n=85)	• 170 infants were enrolled and randomized to the different groups (Control: n=85; Test Group: n=85; Reference Group: n=50) • After randomization, 24 subjects in the Control Group, 25 in Test Group 1, and 5 in the Reference Group either dropped out or were excluded due to a medical condition, adverse event, the ingestion of formula >25% before 6 months in breast-fed infants, or the ingestion of >25% human milk before 3 months in formula fed infants; the distribution and types of adverse events in the different groups were not provided. • No safety parameters reported • Formula fed infants with minor alleles in the fatty acid desaturase genes were associated with declined desaturase activity and lower ARA and DHA levels, regardless of ARA/DHA supplementation • Subsequent subgroup analysis of the COGNIS study described in Nieto-Ruiz et al., 2019; Nieto-Ruiz et al., 2020; Sepulveda-Valbuena et al., 2021; Nieto-Ruiz et al., 2022.

E. ALLERGENICITY

M. alpina is a common soil fungus to which humans are frequently exposed (Streekstra, 1997). *M. alpina* is non-allergenic, non-toxigenic, non-pathogenic, and does not form potentially allergenic spores. A search performed on May 25, 2022, on PubMed using the term "*M. alpina*" and "allergy" yielded no results. Searching for "arachidonic acid" and "allergy" found a report of decreased allergies in infants receiving infant formula supplemented with ARA (Foiles et al., 2016). Therefore, ARA-rich oil is not expected to induce an allergic response.

F. REGULATORY APPROVALS ACROSS THE WORLD

ARA-rich oils derived by fermentation of the fungus *M. alpina* have been used in commercially available infant formulas in at least 50 countries since the early 1990s. They are considered safe for use in infant formula in the United States (GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2008), and GRN 80/41 (2001)), have been the subject of extensive safety reviews conducted by the European Food Safety Authority and the Food Standards Australia New Zealand (EFSA, 2008; FSANZ 2003), and are considered novel foods in Australia, New Zealand, and the European Union.

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

Almaas AN, Tamnes CK, Nakstad B, Henriksen C, Grydeland H, Walhovd KB, Fjell AM, Iversen PO, Drevon CA. Diffusion tensor imaging and behavior in premature infants at 8 years of age, a randomized controlled trial with long-chain polyunsaturated fatty acids. Early Hum Dev. 2016 Apr;95:41-6. doi: 10.1016/j.earlhumdev.2016.01.021. Epub 2016 Mar 2.

Almaas AN, Tamnes CK, Nakstad B, Henriksen C, Walhovd KB, Fjell AM, Due-Tønnessen P, Drevon CA, Iversen PO. Long-chain polyunsaturated fatty acids and cognition in VLBW infants at 8 years: an RCT. Pediatrics. 2015 Jun;135(6):972-80. doi: 10.1542/peds.2014-4094. Epub 2015 May 18.

American Academy of Pediatrics Policy, 2012. Policy Statement, Breastfeeding and the Use of Human Milk. Pediatrics 2012; 129:e827–e841.

Arterburn LM, Boswell KD, Lawlor T, Cifone MA, Murli H, Kyle DJ. In vitro genotoxicity testing of ARASCO and DHASCO oils. Food Chem Toxicol. 2000 Nov;38(11):971-6. doi: 10.1016/s0278-6915(00)00085-5. PMID: 11038233.

Bajpai PK, Bajpai P, Ward OP. Production of arachidonic acid by Mortierella alpina ATCC 32222. J Ind Microbiol. 1991 Oct;8(3):179-85.

Birch EE, Carlson SE, Hoffman DR, Fitzgerald-Gustafson KM, Fu VL, Drover JR, Castañeda YS, Minns L, Wheaton DK, Mundy D, Marunycz J, Diersen-Schade DA. The DIAMOND (DHA Intake And Measurement Of Neural Development) Study: a double-masked, randomized controlled clinical trial of the maturation of infant visual acuity as a function of the dietary level of docosahexaenoic acid. Am J Clin Nutr. 2010;91:848-859.

Birch EE, Garfield S, Castañeda Y, Hughbanks-Wheaton D, Uauy R, Hoffman D. Visual acuity and cognitive outcomes at 4 years of age in a double-blind, randomized trial of long-chain polyunsaturated fatty acid-supplemented infant formula. Early Hum Dev. 2007 May;83(5):279-84. Epub 2007 Jan 18.

Birch EE, Castañeda YS, Wheaton DH, Birch DG, Uauy RD, Hoffman DR. Visual maturation of term infants fed long-chain polyunsaturated fatty acid-supplemented or control formula for 12 mo. Am J Clin Nutr. 2005 Apr;81(4):871-9.

Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. Am J Clin Nutr. 2007 Jun;85(6):1457-64.

Carnielli VP, Simonato M, Verlato G, Luijendijk I, De Curtis M, Sauer PJ, Cogo PE. Synthesis of long-chain polyunsaturated fatty acids in preterm newborns fed formula with long-chain polyunsaturated fatty acids. Am J Clin Nutr. 2007 Nov;86(5):1323-30.

Casterton PL, Curry LL, Lina BA, Wolterbeek AP, Kruger CL. 2009.90-Day feeding and genotoxicity studies on a refined arachidonic acid-rich oil. Food Chem Toxicol. 2009 Oct;47(10):2407-18

Clandinin MT, Van Aerde JE, Merkel KL, Harris CL, Springer MA, Hansen JW, Diersen-Schade DA. Growth and development of preterm infants fed infant formulas containing docosahexaenoic acid and arachidonic acid. J Pediatr. 2005 Apr;146(4):461-8.

Colombo J, Carlson SE, Cheatham CL, Fitzgerald-Gustafson KM, Kepler A, Doty T. Long-chain polyunsaturated fatty acid supplementation in infancy reduces heart rate and positively affects distribution of attention. Pediatr Res. 2011 Oct;70(4):406-10. doi: 10.1203/PDR.0b013e31822a59f5.

Deacon JW. Fungal Biology, 4th Edition. 2006, pg 23, Blackwell Publishing, Oxford, UK.

Domsch KH, Gams W, Anderson TH, Compendium of soil fungi. Volume 1, 1980, pp 259-260, Academic Press, London.

Drover JR, Felius J, Hoffman DR, Castañeda YS, Garfield S, Wheaton DH, Birch EE. A randomized trial of DHA intake during infancy: school readiness and receptive vocabulary at 2-3.5 years of age. Early Hum Dev. 2012 Nov;88(11):885-91. doi: 10.1016/j.earlhumdev.2012.07.007. Epub 2012 Jul 25.

Drover JR, Hoffman DR, Castañeda YS, Morale SE, Garfield S, Wheaton DH, Birch EE. Cognitive function in 18-month-old term infants of the DIAMOND study: a randomized, controlled clinical trial with multiple dietary levels of docosahexaenoic acid. Early Hum Dev. 2011 Mar;87(3):223-30. doi: 10.1016/j.earlhumdev.2010.12.047. Epub 2011 Feb 3.

EFSA (European Food Safety Authority). 2008. Scientific Opinion of the Panel on Dietetic Products Nutrition and Allergies on a request from the European Commission on the safety of 'fungal oil from *Mortierella alpina*'. EFSA Journal 770: 1-15.

European Union (Commission Regulation (EU) 2020/1322 of 23 September 2020 amending Regulation (EC) No 1881/2006 as regards maximum levels of 3-monochloropropanediol (3-MCPD), 3-MCPD fatty acid esters and glycidyl fatty acid esters in certain foods, Official Journal of the European Union, https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020R1322&rid=.

Falk MC, Zheng X, Chen D, Jiang Y, Liu Z, Lewis KD. Developmental and reproductive toxicological evaluation of arachidonic acid (ARA)-Rich oil and docosahexaenoic acid (DHA)-Rich oil. Food Chem Toxicol. 2017 May;103:270-278. doi: 10.1016/j.fct.2017.03.011. Epub 2017 Mar 8. PMID: 28284730.

Floris LM, Stahl B, Abrahamse-Berkeveld M, Teller IC. Human milk fatty acid profile across lactational stages after term and preterm delivery: A pooled data analysis. Prostaglandins Leukot Essent Fatty Acids. 2020 May;156:102023. doi: 10.1016/j.plefa.2019.102023. Epub 2019 Oct 16. PMID: 31699594.

Foiles AM, Kerling EH1, Wick JA, Scalabrin DM, Colombo J, Carlson SE. Formula with long-chain polyunsaturated fatty acids reduces incidence of allergy in early childhood. Pediatr Allergy Immunol. 2016 Mar;27(2):156-61. doi: 10.1111/pai.12515. Epub 2016 Jan 21.

FSANZ, 2003. DHASCO and ARASCO Oils as Sources of Long-Chain Polyunsaturated Fatty Acids in Infant Formula, A Safety Assessment, Technical Report Series No. 22. Food Standards Australia New Zealand 2003.

Gao Y, Li C, Kang L, Hang B, Yan M, Li S, Jin H, Lee AW, Cho SS. A subchronic toxicity study, preceded by an in utero exposure phase, with refined arachidonic acid-rich oil (RAO) derived from Mortierella alpina XM027 in rats. Regul Toxicol Pharmacol. 2014 Dec;70(3):696-703. doi: 10.1016/j.yrtph.2014.10.009. Epub 2014 Oct 24.

Groh-Wargo S, Jacobs J, Auestad N, O'Connor DL, Moore JJ, Lerner E. Body composition in preterm infants who are fed long-chain polyunsaturated fatty acids: a prospective, randomized, controlled trial. Pediatr Res. 2005 May;57(5 Pt 1):712-8. Epub 2005 Feb 17.

Hadley KB, Ryan AS, Forsyth S, Gautier S, Salem N Jr. The Essentiality of Arachidonic Acid in Infant Development. Nutrients. 2016 Apr 12;8(4):216. doi: 10.3390/nu8040216.

Hempenius R, JMH van Delft, M Prinsen and BAR Lina. 1997. Preliminary safety assessment of an arachidonic acid-enriched oil derived from *Mortierella alpina:* Summary of toxicological data. Food and Chemical Toxicology 35: 573-581.

Hempenius RA, BAR Lina and RC Haggit. 2000. Evaluation of a sub-chronic (1 3-week) oral toxicity study, preceded by an in utero exposure phase, with arachidonic acid oil derived from *Mortierella alpina* in rats. Food and Chemical Toxicology 38: 127-139.

Henriksen C, Haugholt K, Lindgren M, Aurvåg AK, Rønnestad A, Grønn M, Solberg R, Moen A, Nakstad B, Berge RK, Smith L, Iversen PO, Drevon CA. Improved cognitive development among preterm infants attributable to early supplementation of human milk with docosahexaenoic acid and arachidonic acid. Pediatrics. 2008 Jun;121(6):1137-45. doi: 10.1542/peds.2007-1511. PMID: 18519483.

Hirose D, Shirouzu T, Hirota M, Ohtsuka T, Senga Y, Du M, Shimono A, Zhang X, Tang Y. Comparison of fungal communities associated with the decomposition of cotton strips among the altitudes on the Tibetan Plateau. Unpublished, submitted to NCBI Aug 20, 2009.

Koletzko B, Lien E, Agostoni C, Böhles H, Campoy C, Cetin I, Decsi T, Dudenhausen JW, Dupont C, Forsyth S, Hoesli I, Holzgreve W, Lapillonne A, Putet G, Secher NJ, Symonds M, Szajewska H, Willatts P, Uauy R; World Association of Perinatal Medicine Dietary Guidelines Working Group. The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. J Perinat Med. 2008;36(1):5-14. doi: 10.1515/JPM.2008.001. PMID: 18184094.

Koletzko B, Boey C, C, M, Campoy C, Carlson S, E, Chang N, Guillermo-Tuazon M, A, Joshi S, Prell C, Quak S, H, Sjarif D, R, Su Y, Supapannachart S, Yamashiro Y, Osendarp S, J, M: Current Information and Asian Perspectives on Long-Chain Polyunsaturated Fatty Acids in Pregnancy, Lactation, and Infancy: Systematic Review and Practice Recommendations from an Early Nutrition Academy Workshop. Ann Nutr Metab 2014;65:49-80. doi: 10.1159/000365767

Koletzko B, Bergmann K, Brenna JT, Calder PC, Campoy C, Clandinin MT, Colombo J, Daly M, Decsi T, Demmelmair H, Domellöf M, FidlerMis N, Gonzalez-Casanova I, van Goudoever JB, Hadjipanayis A, Hernell O, Lapillonne A, Mader S, Martin CR, Matthäus V, Ramakrishan U, Smuts CM, Strain SJJ, Tanjung C, Tounian P, Carlson SE. Should formula for infants provide arachidonic acid along with DHA? A position paper of the European Academy of Paediatrics and the Child Health Foundation. Am J Clin Nutr. 2020 Jan 1;111(1):10-16. doi: 10.1093/ajcn/nqz252. PMID: 31665201.

Koletzko B, Lapillonne A. Lipid Requirements of Preterm Infants. World Rev Nutr Diet. 2021;122:89-102. doi: 10.1159/000514749. Epub 2021 Aug 5. PMID: 34352765.

Koskelo EK, Boswell K, Carl L, Lanoue S, Kelly C, Kyle D. High levels of dietary arachidonic acid triglyceride exhibit no subchronic toxicity in rats. Lipids. 1997 Apr;32(4):397-405. doi: 10.1007/s11745-997-0051-y. PMID: 9113628.

Kremmyda LS, Tvrzicka E, Stankova B, Zak A. Fatty acids as biocompounds: their role in human metabolism, health and disease: a review. part 2: fatty acid physiological roles and applications in human health and disease. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2011 Sep;155(3):195-218. doi: 10.5507/bp.2011.052.

Kroes R, Schaefer EJ, Squire RA, Williams GM. A review of the safety of DHA45-oil. Food Chem Toxicol. 2003 Nov;41(11):1433-46.

Lewis KD, Huang W, Zheng X, Jiang Y, Feldman RS, Falk MC. Toxicological evaluation of arachidonic acid (ARA)-rich oil and docosahexaenoic acid (DHA)-rich oil. Food Chem Toxicol. 2016 Oct;96:133-44. doi: 10.1016/j.fct.2016.07.026. Epub 2016 Jul 25.

Lina BAR, Wolterbeek APM, Suwa Y, Fujikawa S, Ishikura Y, Tsuda S, Dohnalek M. 2006. Subchronic (13-week) oral toxicity study, preceded by an in *utero* exposure phase, with arachidonate-enriched triglyceride oil (SUNTGA40S) in rats. Food and Chemical Tox. 44: 326-335.

Lindberg AM and G Molin. 1993. Effect of temperature and glucose supply on the production of polyunsaturated fatty acids by the fungus *Mortierella alpina* CBS 343.66 in fermentor cultures. Appl Microbiol Biotechnol. 39:450-455.

Mackenzie DA, Wongwathanarat P, Carter AT, Archer DB. Isolation and use of a homologous histone H4 promoter and a ribosomal DNA region in a transformation vector for the oil-producing fungus Mortierella alpina Appl. Environ. Microbiol. 66 (11), 4655-4661 (2000).

Martin CR, Dasilva DA, Cluette-Brown JE, Dimonda C, Hamill A, Bhutta AQ, Coronel E, Wilschanski M, Stephens AJ, Driscoll DF, Bistrian BR, Ware JH, Zaman MM, Freedman SD. Decreased postnatal docosahexaenoic and arachidonic acid blood levels in premature infants are associated with neonatal morbidities. J Pediatr. 2011 Nov;159(5):743-749.e1-2. doi: 10.1016/j.jpeds.2011.04.039. Epub 2011 Jun 12.

Martin JC, Bougnoux P, Antoine JM, Lanson M, Couet C. Triacylglycerol structure of human colostrum and mature milk. Lipids. 1993 Jul;28(7):637-43.

Martinez JA, Ballew MP. Infant formulas. Pediatr Rev. 2011 May;32(5):179-89; quiz 189. doi: 10.1542/pir.32-5-179. PMID: 21536776.

Mendonça MA, Araújo WMC, Borgo LA, Alencar ER. Lipid profile of different infant formulas for infants. PLoS One. 2017 Jun 1;12(6):e0177812. doi: 10.1371/journal.pone.0177812. PMID: 28570611; PMCID: PMC5453432.

Merritt RJ, Auestad N, Kruger C, Buchanan S. 2003. Safety evaluation of sources of docosahexaenoic acid and arachidonic acid for use in infant formulas in newborn piglets. Food and Chemical Tox. 41: 897-904.

Nes W and Nichols S. (2006). Phytosterol biosynthesis pathway in *Mortierella alpina*, Phytochemistry, 67(16):1716-1721.

Nieto-Ruiz A, García-Santos JA, Verdejo-Román J, Diéguez E, Sepúlveda-Valbuena N, Herrmann F, Cerdó T, De-Castellar R, Jiménez J, Bermúdez MG, Pérez-García M, Miranda MT, López-Sabater MC, Catena A, Campoy C. Infant Formula Supplemented With Milk Fat Globule Membrane, Long-Chain Polyunsaturated Fatty Acids, and Synbiotics Is Associated With Neurocognitive Function and Brain Structure of Healthy Children Aged 6 Years: The COGNIS Study. Front Nutr. 2022 Mar 9;9:820224. doi: 10.3389/fnut.2022.820224. PMID: 35356726; PMCID: PMC8959863.

Nieto-Ruiz A, Diéguez E, Sepúlveda-Valbuena N, Herrmann F, Cerdó T, López-Torrecillas F, De-Castellar R, Jiménez J, Pérez-García M, Miranda MT, Catena A, García-Santos JA, Bermúdez MG, Campoy C. The Effects of an Infant Formula Enriched with Milk Fat Globule Membrane, Long-Chain Polyunsaturated Fatty Acids and Synbiotics on Child Behavior up to 2.5 Years Old: The COGNIS Study. Nutrients. 2020 Dec 15;12(12):3825. doi: 10.3390/nu12123825. PMID: 33333767; PMCID: PMC7765166.

Nieto-Ruiz A, García-Santos JA, Bermúdez MG, Herrmann F, Diéguez E, Sepúlveda-Valbuena N, García S, Miranda MT, De-Castellar R, Rodríguez-Palmero M, Catena A, Campoy C. Cortical Visual Evoked Potentials and Growth in Infants Fed with Bioactive Compounds-Enriched Infant Formula: Results from COGNIS Randomized Clinical Trial. Nutrients. 2019 Oct 14;11(10):2456. doi: 10.3390/nu11102456. PMID: 31615134; PMCID: PMC6835488.

Orsavova J, Misurcova L, Ambrozova JV, Vicha R, Mlcek J. Fatty Acids Composition of Vegetable Oils and Its Contribution to Dietary Energy Intake and Dependence of Cardiovascular Mortality on Dietary Intake of Fatty Acids. Int J Mol Sci. 2015 Jun 5;16(6):12871-90. doi: 10.3390/ijms160612871. PMID: 26057750; PMCID: PMC4490476.

Salas Lorenzo I, Chisaguano Tonato AM, de la Garza Puentes A, Nieto A, Herrmann F, Dieguez E, Castellote AI, López-Sabater MC, Rodríguez-Palmero M, Campoy C. The Effect of

an Infant Formula Supplemented with AA and DHA on Fatty Acid Levels of Infants with Different FADS Genotypes: The COGNIS Study. *Nutrients*. 2019; 11(3):602. https://doi.org/10.3390/nu11030602.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA. Chen W. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi Proc. Natl. Acad. Sci. U.S.A. 109(16):6241-6 (2012).

Scholer HJ, Miiller E, Schipper MAA. Mucorales, in Fungi pathogenic for humans and animals, Marcel Dekker, New York, 1983, pp 9-24.

Sekiguchi H, Masunaka A, Hashimoto Y, and Takenaka S. Soil Microbial Community Affect Colonization of Bio-control Agent Pythium oligandrum in Micro-tom Rhizosphere. Unpublished, direct submission to NCBI Jan 8, 2009.

Sepúlveda-Valbuena N, Nieto-Ruiz A, Diéguez E, Herrmann F, Escudero-Marín M, De-Castellar R, Rodríguez-Palmero M, Miranda MT, García-Santos JA, Bermúdez MG, Campoy C. Growth patterns and breast milk/infant formula energetic efficiency in healthy infants up to 18 months of life: the COGNIS study. Br J Nutr. 2021 Dec 28;126(12):1809-1822. doi: 10.1017/S000711452100057X. Epub 2021 Feb 19. PMID: 33602357.

Shimizu S, Kawashima H, Wada M, Yamada H. (1992). Occurrence of a novel sterol, 24, 25-methylenecholest-5-en-3β-ol, in *Mortierella alpina* 1S-4. Lipids, 27(6): 481-483.

Shinmen Y, Shimizu S, Akimoto K, Kawashima H, Yamada H. Production of arachidonic acid by Mortierella fungi. Appl Microbiol Biotechnol (1989) 31:11-16.

Streekstra H. On the safety of *Mortierella alpina* for the production of food ingredients, such as arachidonic acid. J Biotechnol. 1997;56:153-165.

Tagawa M, Tamaki H, Manome A, Koyama O, Kamagata Y. Isolation and characterization of antagonistic fungi against potato scab pathogens from potato field soils. FEMS Microbiol. Lett. 305 (2), 136-142 (2010).

Takada Hoshino Y, Morimoto S. Soil Clone Library Analyses to Evaluate Specificity and Selectivity of PCR Primers Targeting Fungal 18S rDNA for Denaturing-Gradient Gel Electrophoresis (DGGE)Microbes Environ. 25 (4), 281-287 (2010).

Tan L, Ma F, Li S. 18S rDNA and internal transcribed spacer polymerase chain reaction primers for identification of a single cell oil-producing fungi. Unpublished, direct submission to NCBI Apr 24, 2014.

Totani N, Oba K. The Filamentous Fungus *Mortierella alpina*, High in Arachidonic Acid. Lipids 22, 1060-1062, 1987.

Tsuji M, Tanabe Y, Uetake J. D1/D2 domain sequence of psychrophilic fungi from Arctic biological soil cluster. Unpublished, submitted to NCBI Feb 9, 2016.

van Brakel L, Thijs C, Mensink RP, Lütjohann D, Plat J. Non-Cholesterol Sterols in Breast Milk and Risk of Allergic Outcomes in the First Two Years of Life. Nutrients. 2022 Feb 11;14(4):766. doi: 10.3390/nu14040766. PMID: 35215415; PMCID: PMC8874767.

Wagner L, Stielow B, Hoffmann K, Petkovits T, Papp T, Vágvölgyi C, de Hoog GS, Verkley G, Voigt K. A comprehensive molecular phylogeny of the Mortierellales (Mortierellomycotina) based on nuclear ribosomal DNA. Persoonia. 2013 Jun;30:77-93. doi: 10.3767/003158513X666268. Epub 2013 Mar 13.

Westerberg AC, Schei R, Henriksen C, Smith L, Veierød MB, Drevon CA, Iversen PO. Attention among very low birth weight infants following early supplementation with docosahexaenoic and arachidonic acid. Acta Paediatr. 2011 Jan;100(1):47-52. doi: 10.1111/j.1651-2227.2010.01946.x.

Xu B, Zhang L, Ma F, Zhang W, Wang X, Zhang Q, Luo D, Ma H, Li P. Determination of free steroidal compounds in vegetable oils by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry. Food Chem. 2018 Apr 15;245:415-425. doi: 10.1016/j.foodchem.2017.10.114. Epub 2017 Oct 25. PMID: 29287390.

Yang R, Xue L, Zhang L, Wang X, Qi X, Jiang J, Yu L, Wang X, Zhang W, Zhang Q, Li P. Phytosterol Contents of Edible Oils and Their Contributions to Estimated Phytosterol Intake in the Chinese Diet. Foods. 2019 Aug 9;8(8):334. doi: 10.3390/foods8080334. PMID: 31404986; PMCID: PMC6723959.

B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of Hubei Fuxing's ARA-rich oil for the intended use specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b).

Hubei Fuxing Biotechnology Co., Ltd is proposing to market ARA-rich oil, produced by Hubei Fuxing Biotechnology Co., Ltd, China, as a source of ARA-rich oil used in the manufacture of infant formula. The end-use infant formulas are exempt pre-term infant formula and non-exempt term infant formula. Consistent with other GRAS sources of ARA-rich oil GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001) and GRN 41 (2001), this ingredient is produced by the fungus *Mortierella alpina* and specifications stipulate a minimum of 40% arachidonic acid in the oil.

The following safety evaluation considers the composition, intake, nutritional, microbiological, and toxicological properties of Hubei Fuxing's ARA-rich oil based on publicly available data from essentially equivalent ARA-rich oils as determined GRAS in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001) and GRN 41 (2001), each of which received "no questions" letters from the United States Food and Drug Administration (FDA). The proposed use of Hubei Fuxing's ARA-rich oil as an ingredient in non-exempt term infant formula and exempt pre-term infant formula has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based upon the following:

- The compositional data and product specifications are demonstrative of carefully controlled production and refining processes.
- The morphology, biochemistry, and physiology of *M. alpina* are well-documented and it is not pathogenic or toxic. The *M. alpina* strain used in the production of ARA-rich oil is not genetically modified.
- The FDA has issued 'no question' letters for six GRAS notices for ARA-rich oils derived from *M. alpina* for infant formula: GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001). A comparison of the specifications among the ARA-rich oil that is the subject of this notification and those in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001) demonstrate that the product specifications for Hubei

Fuxing's ARA-rich oil are comparable to the product specifications for the ARA-rich oil generated from *M. alpina* as described in those GRNs, with some parameters being more stringently controlled, including acid value, anisidine value, mercury, and moisture. Specifications for ARA-rich oils determined GRAS in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001) show that they are similar in composition to the ARA rich oil produced by Hubei Fuxing. Accordingly, the information described in these ARA-rich oil GRNs is relevant as supportive data for the subject of this Notice.

- The intended use for ARA-rich oil is as an ingredient in exempt and non-exempt infant formulas. The functional importance of long-chain polyunsaturated fatty acids (LCPUFA) in pregnancy, lactation, and infancy has been the subject of numerous clinical evaluations, particularly as they relate to the intake of the n-6 and n-3 LCPUFAs, ARA (C20:4n-6) and docosahexaenoic acid (DHA; C22:6n-3). Studies indicate that infants may not synthesize sufficient amounts of ARA and DHA *de novo* from their respective precursors to cover the high demand during this period of rapid accretion for critical normal growth and development. Because breastfeeding and human milk are the normative standards for infant feeding and nutrition (American Academy of Pediatrics Policy, 2012), infant formula must be capable of supporting the nutritional needs of pre-term and term infants when infant formula is chosen as a surrogate for human milk. Based on current knowledge regarding the importance of LCPUFA in infant nutrition, their presence in human milk, and the guidelines and recommendations established by the European Academy of Paediatrics, World Association of Perinatal Medicine, the Early Nutrition Academy, and the Child Health Foundation, infant formula should contain 0.3 to 0.5 weight percent of total dietary fat, with the minimum amount of ARA being equivalent to the DHA content.

- The intended use of ARA-rich oil is to provide a source of ARA in infant formula at a concentration consistent with that of human milk. The proposed use of ARA-rich oil is intended to provide 0.75% and 0.40% ARA by weight of fatty acids in term and pre-term infant formulas, respectively. This is within the range of ARA found in human milk. The intended use of ARA-rich oil to deliver this concentration of ARA corresponds to 1.875% of total fat for non-exempt term infant formula and 1.0% of total fat for exempt pre-term infant formula. This intended use level is consistent with the levels of use currently recommended by the European Academy of Paediatrics and the Child Health Foundation.

- An estimate of exposure to ARA from its addition to infant formula at target ARA levels of 0.75 g and 0.40 g per 100 g total fat for term and pre-term infant formulas may be calculated as follows: assuming human infants consume 100 kcal/kg body weight/day (term infants) to 120 kcal/kg body weight/day (pre-term infants), of which fat comprises about 50% of the energy, an infant will consume 5.6 g (term infants) to 6.7 g (pre-term infants) of fat/kg body weight/day (1 g fat = 9 kcal). These amounts correspond to intakes of ARA of 42 mg and 27 mg ARA/kg body weight/day (or 105 and 67 mg of ARA-rich oil/kg body weight/day) for term infants and pre-term infants, respectively.

- The source organism, manufacturing process, product specifications, and intended uses of Hubei Fuxing's ARA-rich oil are essentially equivalent to ARA-rich oil cited in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001); therefore, publicly available animal and human safety and tolerance studies of this ARA-rich oil support the safety of Hubei Fuxing's ARA-rich oil.

    - The safety of ARA-rich oils as ingredients in infant formula has been reviewed by numerous regulatory bodies worldwide. In these jurisdictions, the conclusions reached were that ARA-rich oils derived from *M. alpina*, meeting appropriate food grade specifications, provide a safe ARA source for supplementation of infant formula. These decisions have led to its availability for this use in at least 50 countries worldwide.

- Numerous in vitro and in vivo safety studies have been conducted over a period of more than two decades on ARA-rich oils derived from *M. alpina*. ARA-rich oil is not genotoxic as assessed by multiple genotoxicity assay. The toxicology studies cited in GRN 963 (2021), GRN 730 (2018), GRN 326 (2010), GRN 94 (2006), GRN 80 (2001), and GRN 41 (2001) include four subchronic toxicology studies with an *in utero* exposure which establish a range of no-observed adverse event levels (NOAELs) of 1.5-5% ARA-rich oil in the diet, or 374-3170 mg/kg/day (Hempenius, Lina, and Haggitt 2000; Casterton et al. 2009; Gao et al. 2014; Lina et al. 2006). Additional toxicology studies showed a lack of adverse effects on developmental or reproductive parameters. Tolerance and safety in a neonatal piglet model determined that dietary ARA concentration of up to 96 mg ARA/100 kcal was safe and well tolerated.

    - A corroborative, unpublished bacterial reverse mutation assay of Hubei Fuxing's ARA-rich oil was negative.

- There were no test article-related adverse effects reported in clinical studies of infant formula containing fungal-derived ARA-rich oils in pre-term infants when used at levels up to 0.91% total fatty acid content.
- Clinical studies, detailed in GRN 963 (2021) and GRN 326 (2010) using 0.64-0.72% of total fatty acids as ARA also confirmed the safety of infant formula containing fungal-derived ARA-rich oil in term and preterm infants.

Taken together, the available data from studies conducted on ARA-rich oils from *M. alpina* establish a strong body of evidence for the safety of ARA-rich oil as a source of ARA for supplementation of infant formula. ARA-rich oil is safe and GRAS at the proposed levels of ingestion. It is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

Roger Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
School of Pharmacy
University of Southern California

Signature: 
Date: September 7, 2022

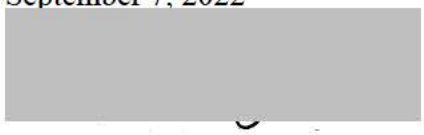
A. Wallace Hayes, PhD, DABT, FATS, ERT
GRAS Expert Panel Member
University of South Florida College of Public Health

Signature: 
Date: September 7, 2022

Thomas E. Sox, PhD, JD
GRAS Expert Panel Member
Principal, Pondview Consulting LLC

Signature: 
Date: September 7, 2022

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel
Spherix Consulting Group, Inc.

Signature: 
Date: September 7, 2022

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001115	Sept 27, 2022
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*
   ☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 08/24/2022

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd)*: _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Rebecca Lee	Export Manager

Organization *(if applicable)*: Hubei Fuxing Biotechnology Co., Ltd.

Mailing Address *(number and street)*: No. 18 Fuxing Ave, Chenhu Town

City	State or Province	Zip Code/Postal Code	Country
Hanchuan City	Hubei Province	431608	China

Telephone Number	Fax Number	E-Mail Address
86-27-83660037		fuxing_rebecca@163.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Claire Kruger	Managing Partner

Organization *(if applicable)*: Spherix Consulting Group, Inc.

Mailing Address *(number and street)*: 751 Rockville Pike, Unit 30-B

City	State or Province	Zip Code/Postal Code	Country
Rockville	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
301-775-9476		ckruger@spherixgroup.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Arachidonic acid (ARA)-rich oil

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 963

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRNs 730, 326, 94, 80, and 41

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

The intended use of the subject of this GRAS Notice is to provide a source of ARA in term and pre-term infant formulas at a concentration consistent with that of human milk and the current guidelines regarding safe infant feeding practices. The ARA content of human milk varies from 0.34-1.22% of total fatty acids among different populations. The proposed use of ARA-rich oil is to provide 0.75% and 0.40% ARA by weight of fatty acids in term and pre-term infant formulas, respectively, which is equivalent to 1.875% of total fat in non-exempt term infant formula and 1.0% of total fat in exempt pre-term infant formula. Additionally, the subject of this GRAS Notice will be used in formulas for term and pre-term infants with a safe and suitable source of DHA at levels that comply with current recommendations and guidelines.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Hubei Fuxing Biotechnology Co., Ltd.
(name of notifier)

has concluded that the intended use*(s)* of Arachidonic acid (ARA)-rich oil
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Hubei Fuxing Biotechnology Co., Ltd. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

No. 18 Fuxing Ave, Chenhu Town, Hanchuan City 431608, Hubei Province, China
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Claire L. Kruger, PhD Digitally signed by Claire L. Kruger, PhD Date: 2022.09.21 10:09:55 -04'00'	Claire Kruger, Managing Partner	09/21/2022

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	H-F ARA-Rich Oil GRAS 9-21-22 - To FDA.pdf	Submission
	All References	Submission

From: kbrailer@spherixgroup.com
To: Morissette, Rachel
Cc: "Claire Kruger"; "Dietrich Conze"; "Jennifer Symonds"
Subject: [EXTERNAL] GRN 001115 Response
Date: Thursday, August 3, 2023 3:57:59 PM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png
Response to FDA"s Request for Information on GRN 001115 8-3-23.pdf
Attachment 2 - GB4789.40-2016EN.pdf
Leman 2009 book chapter.pdf
Xu et al 2018 (1).pdf
Attachment 1 - Aluminum Bottles FDA Letter.pdf
Casterton 2009.pdf

Dear Rachel,

Attached please find our response to FDA's questions on GRN 001115. Please confirm receipt and let us know if you need anything else.

Best regards,

Kathy Brailer
Director of Administrative Services
Spherix Consulting Group, Inc.
751 Rockville Pike, Unit 30-B
Rockville, MD 20852
+1-301-557-0375
kbrailer@spherixgroup.com
www.spherixgroup.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, July 20, 2023 8:44 AM
To: Jennifer Symonds <jsymonds@spherixgroup.com>
Cc: Kathy Brailer <kbrailer@spherixgroup.com>; Claire Kruger <ckruger@spherixgroup.com>; Dietrich Conze <dconze@spherixgroup.com>
Subject: RE: [EXTERNAL] Extension for GRN 1115 response

Dear Jennifer,

Thank you. August 4 will be fine.

Best,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From: Jennifer Symonds <jsymonds@spherixgroup.com>
Sent: Friday, July 14, 2023 3:49 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Kathy Brailer <kbrailer@spherixgroup.com>; Claire Kruger <ckruger@spherixgroup.com>; Dietrich Conze <dconze@spherixgroup.com>
Subject: [EXTERNAL] Extension for GRN 1115 response

Hello Rachel,

Thank you for meeting us today. As discussed earlier, we would like to request an extension for our response to the FDA's questions. We propose the new deadline for our response to be August 4th. Those extra weeks will allow us ample time to correspond with our client. Thank you.

Best regards,

Jenn Symonds

--
Jennifer M. Symonds, Ph.D.
Senior Project Manager
Spherix Consulting Group, Inc.
751 Rockville Pike

Unit 30-B
Rockville, MD 20852
+1-319-621-6154

Spherix Consulting Group

August 3, 2023

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive, HFS-200
College Park, MD 20740

Dear Dr. Morissette:

The following are our responses to your questions regarding GRN 001115, as described in your letter dated July 5, 2023. Your requests are in italicized text and our responses are below in plain text. As suggested in your letter, the subject of GRN 001115 will be referred to as "*M. alpina* oil" in our responses and our correspondence moving forward.

Chemistry:

1. *We note that the intended use is described as both an "ingredient" and as a "source of ARA" in the notice. Please clarify the intended use.*

   M. alpina oil is a source of ARA and intended to be used as an ingredient in infant formula.

2. *Hubei Fuxing states that M. alpina oil will be used in combination with a safe and suitable source of docosahexaenoic acid (DHA) and consistently with generally accepted and current guidelines for safe infant feeding practices. Please confirm that the use of M. alpina oil, in accordance with current recommendations, will provide a ratio ranging from 1:1 to 2:1 ARA:DHA in infant formula.*

   The subject of GRN 001115 will be used in accordance with current recommendations, with a safe and suitable source of DHA at a ratio ranging from 1:1 to 2:1 ARA:DHA.

3. *Hubei Fuxing states that the intended uses are substitutional for other sources of ARA in infant formula (i.e., GRNs 000326 and 000963). However, Hubei Fuxing also states (first bullet, p.4) that the intended uses of M. alpina oil are essentially equivalent to those for M. alpina oil cited in GRNs 000963, 000730, 000326, 000094, 000080, and 000041. While it is appropriate to state that the intended uses are substitutional for other M. alpina oils described in these notices, the use levels are not the same for all notices (e.g., GRNs 000041 and 000094 describe lower use levels); therefore, the current uses are not equivalent to earlier GRNs. Please clarify.*

751 Rockville Pike, Unit 30-B
Rockville, MD 20852
301-557-0375; info@spherixgroup.com
http://www.spherixgroup.com

The intended uses are substitutional for other sources of ARA and will be used at the same use levels as the most recent GRAS Notice, GRN 000963 (2021), to receive a "no questions" letter from the Agency.

4. *Please provide additional information about the manufacturing step described as the "solvent leaching process" (p.12), including how residual levels of solvent are minimized.*

The boiling point of butane is -1°; thus, butane is removed from the crude oil by heating the oil to 60°C under negative pressure for one hour during the solvent leaching step, which is repeated 5-6 times. Additionally, any remaining butane in the crude oil is removed during the steam deodorization step, which occurs at 170-190°C. Finally, butane levels are controlled in the final product with the residual solvent product specification of less than or equal to 1 mg/kg.

5. *Please provide additional information about the manufacturing steps that ensure removal of the biomass from the crude oil (p.12), including an explanation for the level of residual biomass (up to 5%).*

The specification of not more than 5% biomass is set for the crude oil as a quality control point to ensure that the biomass in the crude oil does not interfere with the subsequent refining steps. Importantly, approximately 99% of the solids, including the *M. alpina* biomass, are removed from the crude oil using a stainless steel 50-mesh filter. Additionally, any remaining biomass is then removed during the refining process, which involves treating the oil with activated carbon and activated kaolin clay and filtering the product with a filter that has a pore size of 0.2 μm.

6. *In our evaluation of the manufacturing method, we look for fermentation media components to be safe and suitable for their intended use, consistent with industry practices, and of a purity suitable for their intended use. Hubei Fuxing provides a statement that the raw materials are food grade and comply with 21 CFR. However, there are regulations cited in Table 3 (p.16) listing processing aids used in the manufacture of M. alpina oil that are related to the specific ingredient but are not applicable to the intended uses described in the notice. For example, we note:*

   a. *The citation of 21 CFR 168.111 dextrose monohydrate refers to the standard of identity for this sweetener but is not a regulation specifying its intended use.*

   b. *The citation provided for yeast extract 21 CFR 184.1983 pertains to use of this ingredient as a flavoring agent and adjuvant as defined in 21 CFR 170.3(o)(12).*

We request that Hubei Fuxing states that all processing aids and raw materials used in the manufacture of M. alpina oil are safe and suitable for their intended uses and, if applicable, are used in accordance with an existing food additive regulation, are GRAS for their intended use, or are the subject of an effective food contact notification.

The dextrose monohydrate and yeast extract are components of the fermentation medium used to the manufacture of the subject of this Notice. We agree that the two 21 CFR citations that were provided in the GRAS Notice are not relevant. Importantly, all processing aids and raw materials used in the manufacture of the subject of this Notice are safe and suitable for their intended uses and, if applicable, are used in accordance with an existing food additive regulation, are GRAS for their intended use, or are the subject of an effective food contact notification.

7. *Please provide a copy or complete citation of the "FDA memo 2012" referenced for use of aluminum bottles for oil storage (Table 3, p.16).*

The information on the aluminum bottles is attached. Included in this information is a letter from the FDA describing the suitability of the aluminum bottles for food contact use.

8. *For the specifications in Table 4 (p.17), please provide a brief description of the analytical methods listed by Guobiao (GB) method number and confirm that the respective methods for each analyte are validated for that purpose.*

Brief descriptions of the analytical methods listed by Guobiao (GB) method number are provided below in Table 1. Each method has been validated for the purpose of assessing compliance with the product specifications for the subject of this Notice. Additionally, the heavy metal detection methods have been updated to reflect the most recent methodology used to quantify mercury, lead, arsenic, cadmium, and copper in *M. alpina* oil.

Table 1. Description of Methods Used to Assess Product Specifications of Hubei Fuxing *M. alpina* Oil		
GB Method Number	**Parameter Tested**	**Brief Method Description**
GB/T 5525-2008, Vegetable Fats and Oils – Method for Identification of Transparency, Odor, and Flavor	Appearance, Taste and Smell	Taste and smell are assessed by a group of people.
GB/T 22460-2008, Animal and Vegetable Fats and Oils – Determination of Lovibond Color	25.4 mm color	Determination of oil color is done with Lovibond colorimeter.
GB 5009.168-2016, Third Method National Food Safety Standard Determination of Fatty Acids in Foods	ARA content	After the methyl esterification of the oil, the different components of the oil are separated by gas chromatography, and the fatty acid components are identified by the flame ionization detector.
GB 5009.236-2016, National Food Safety Standard – Animal and Vegetable, Fats and Oils – Determination of Moisture and Volatile Matter	Moisture	Under the condition of 103°C±2°C, the test sample is heated until the moisture and volatile matter are completely evaporated, and the mass of the sample loss is measured.
GB 5009.229-2016, National Food Safety Standard – Determination of Acid Value in Foods	Acid value	Dissolve the oil sample with an organic solvent, then neutralize the free fatty acid in the titration sample solution with potassium hydroxide or sodium hydroxide standard titration solution, determine the titration end point by the corresponding color change of the indicator, and finally calculate the acid value of the oil sample by the volume of the standard titration solution consumed at the titration end point.
GB 5009.257-2016, National Food Safety Standard – Determination of Trans-fatty Acids in Foodstuffs	Trans Fatty Acids	Methylate the oil sample, and then use a strong polar gas chromatography column to separate the trans fatty acid, then use a flame ionization detector to identify it.
GB/T 15688-2008, Animal and Vegetable Fats and Oils – Determination of Insoluble Impurities Content	Insoluble impurities	Dissolve the sample with an organic solvent, filter it, and use the same solvent to rinse the residue and the filter paper used for filtration. Dry the residue and filter paper at 103°C until the weight of the paper is stable, then record the filter paper weight and calculate the content of incompatible impurities.
GB/T 5535.2-2008, Animal and Vegetable Fats and Oils – Determination of Unsaponifiable Matter	Unsaponifiable matter	Oil and potassium hydroxide ethanol solution are saponified under the condition of boiling and reflux, the unsaponifiable matter is extracted from the saponified liquid with hexane or petroleum ether, the solvent is evaporated, and the residue is dried to constant weight for calculation.
GB/T 24304-2009, Animal and Vegetable Fats and Oils – Determination of Anisidine Value	p-Anisidine value	Dissolve the sample in isooctane, react with the acetic acid solution of p-anisidine, measure the increased absorbance at 350 nm wavelength, and calculate the anisidine value.
GB 5009.227-2016 First Method, National Food Safety Standard – Determination of Peroxide Value in Foods	Peroxide value	The prepared oil sample was dissolved in chloroform and glacial acetic acid, the peroxide in it reacted with potassium iodide to generate iodine, and the precipitated iodine was titrated with sodium thiosulfate standard solution. The amount of peroxide value is expressed by the mass fraction of peroxide equivalent to iodine or the number of millimoles of active oxygen in 1 kg of sample.

Table 1. Description of Methods Used to Assess Product Specifications of Hubei Fuxing *M. alpina* Oil		
GB Method Number	**Parameter Tested**	**Brief Method Description**
GB 5009.262-2016, National Food Safety Standard – Determination of Solvent Residual in Food	Residual solvent	The solvent residue in the sample will diffuse into the gas phase in the airtight container until the dynamic equilibrium of the concentration between the gas phase and the liquid phase can be reached. The solvent residue content in the upper gas phase is detected by headspace gas chromatography, and the actual content of the residual solvent in the sample to be tested is calculated.
GB 5009.17-2021, National Food Safety Standard Determination of Total Mercury and Organic Mercury in Food	Mercury	After the sample is heated and digested by acid, in the acid medium, the mercury in the sample is reduced to atomic mercury by potassium borohydride or sodium borohydride, which is brought into the atomizer by the carrier gas (argon gas), and then released in the mercury hollow cathode lamp. Under irradiation, mercury atoms in the ground state are excited to a high-energy state, and when they return to the ground state from the high-energy state, they emit fluorescence at a characteristic wavelength, and its fluorescence intensity is proportional to the mercury content and is quantified by external standard method.
GB 5009.12-2017, National Food Safety Standard Determination of Lead in Food	Lead	After the sample is digested, it is atomized in a graphite furnace, and the absorbance is measured at 283.3 nm. The absorbance value of lead within a certain concentration range is directly proportional to the lead content, which is quantitative compared with the standard series.
GB 5009.11-2014, National Food Safety Standard Determination of Total Arsenic and Inorganic Arsenic in Food	Arsenic	The sample is processed into a sample solution by acid digestion, and the sample solution is sent into the inductively coupled plasma rectangular tube by atomization from the carrier gas, and is transformed into charged ions through evaporation, dissociation, atomization and ionization, and then passed through the ion collection system, and enters into the mass spectrometer, which separates according to the mass-to-charge ratio. For a certain mass-to-charge ratio, the signal intensity of the mass spectrum is proportional to the number of ions entering the mass spectrometer, that is, the sample concentration is proportional to the signal intensity of the mass spectrometer. The arsenic element in the sample solution was determined by measuring the signal intensity of the mass spectrum.
GB 5009.15-2014, National Food Safety Standard - Determination of Cadmium in Food	Cadmium	After the sample is ashed or digested with acid, a certain amount of sample digestion solution is injected into the graphite furnace of the atomic absorption spectrophotometer, and the 228.8 nm resonance line is absorbed after electrothermal atomization. Within a certain concentration range, the absorbance value is proportional to the cadmium content, quantified by the standard curve method.

Table 1. Description of Methods Used to Assess Product Specifications of Hubei Fuxing *M. alpina* Oil		
GB Method Number	**Parameter Tested**	**Brief Method Description**
GB 5009.13-2017, National Food Safety Standard Determination of Copper in Food	Copper	After the sample is digested, it is atomized in a graphite furnace, and the absorbance is measured at 324.8 nm. In a certain concentration range, the absorbance value of copper is proportional to the copper content, which is quantitative compared with the standard series.
GB 4789.3-2016 Second method, National Food Safety Standard – Food Microbiological Examination – Enumeration of Coliforms	Coliforms	After the sample is serially diluted and cultured, the maximum possible number of coliform bacteria in the sample is deduced by applying statistical probability theory according to the lowest dilution without growth and the highest dilution with growth.
GB 4789.15-2016 First method, National Food Safety Standard – Food Microbiological Examination – Enumeration of Moulds and Yeasts	Molds and Yeast	The sample is inoculated on the medium for cultivation and counted.
GB 4789.4-2016, National Food Safety Standard – Food Microbiological Examination – Salmonella Test	Salmonella	
GB 4789.2-2016, National Food Safety Standard – Food Microbiological Examination of Food Hygiene – Detection of Aerobic Bacterial Count	Aerobic plate count	
GB 4789.40-2016, National Food Safety Standard – Food Microbiological Examination – Examination of Cronobacter (Enterobacter Sakazakii)	*Cronobacter sakazakii*	

9. *In Table 4 (p.17), there is a specification for copper of ≤1.0 mg/kg. We would not expect the refined oil to contain copper, and the batch analyses exhibited non-detectable levels of copper. Please address the basis for including a specification of 1.0 mg/kg for copper.*

   Copper was included as a specification to meet the regulatory requirements of other jurisdictions. Copper is not expected to be present in the finished *M. alpina* oil.

10. *In Table 4 (p.17), trans fatty acids are specified at ≤ 1.0%. However, in Table 9 (p.28), trans fatty acids are listed as "not specified." Please confirm that the specification Hubei Fuxing provides in Table 4 is correct and clarify the units (e.g., percent total fatty acids, total fat, or ingredient basis) for this specification.*

    The specification for trans fatty acids in Table 4 is correct and the unit for specification is % of the total fatty acids.

11. *Regarding data presented in Table 5 (p.18), please clarify if the fatty acid values are expressed on a percentage of total fatty acids, percentage of total fat, or percentage of ingredient (as is) basis.*

    The fatty acid values presented in Table 5 are expressed as percentage of the ingredient, *M. alpina* oil.

12. *Please clarify the following regarding the specification for residual solvent:*

    a. *In Table 9 (p.28), there is a specification of ≤1.0 mg/kg for "residual solvents (butane or hexane)" in M. alpina oil, which is compared to previous GRNs for M. alpina oil. Although GRN 000326 is indicated as the only previous GRN to have set a limit for residual solvent, we note that other notices also included limits for residual hexane (e.g., GRNs 000094, 000730) in tables provided in the respective amendments, or butane in the discussion of the manufacturing and monitored limits (e.g., GRN 000963).*

    b. *In Table 10 (p.30), there is a limit for hexane residues of not more than (NMT) 1.0 mg/kg. Hexane is not mentioned in the manufacturing method, so a specification would not be expected. Please clarify if hexane is used and if this limit should apply to butane.*

       Hexane is not used in the production of the subject of GRN 001115; therefore, a limit for hexane does not apply. The purpose of Table 9 is to compare the specifications among *M. alpina* oils that are GRAS. The entry for residual solvents "(hexane or butane)" was used to be inclusive of the subjects of the other GRAS Notices for *M. alpina* oils that utilize either hexane or butane during production. The purpose of Table 10 is to compare the specifications listed in the Food Chemicals Codex monograph for ARA from Fungal (*Mortierella alpina*) Oil with the specifications for the subject of GRN 001115. Because hexane is not used in the production of the subject of GRN 001115, the entry specified for "Hexane Residues" in the column entitled "Hubei Fuxing ARA-oil Specification" in Table 10 should be "not applicable".

13. *Hubei Fuxing notes on pp.30-31 that palmitic, stearic, oleic, linoleic, linolenic, eicosatrienoic, arachidonic, behenic, and lignoceric fatty acids contribute over 86% of the total fatty acids in all ARA-rich oils and are indicated in bold in Table 11 (pp.32-33). Please confirm that in this statement "linolenic" refers to gamma-linolenic acid (GLA) rather than*

alpha-linolenic acid and that "eicosatrienoic" refers to dihomo-gamma-linolenic acid (DGLA) rather than mead acid.

We confirm that "linolenic" refers to gamma-linolenic acid (GLA) rather than alpha-linolenic acid and that "eicosatrienoic" refers to dihomo-gamma-linolenic acid (DGLA) rather than mead acid.

14. *Hubei Fuxing notes that comparing the fatty acid profile of M. alpina oil to that in the Food Chemicals Codex (FCC) is not relevant given that the fatty acid limits in the FCC are expressed in units of percent area rather than percent total fatty acids or percent of total fat. In lieu of a comparison to the FCC monograph for "ARA from fungal (Mortierella alpina) oil" (13th edition, 2023), please provide specifications for the other fatty acids (palmitic, stearic, oleic, linoleic, GLA, DGLA, behenic, and lignoceric) present in M. alpina oil that also comprise up to 86% of total fatty acids.*

As discussed in our meeting with the review team at FDA on July 14th, the typical range of palmitic, stearic, oleic, linoleic, gamma-linolenic, dihomo-gamma linolenic, behenic, and lignoceric acids found in the subject of GRN 001115 is shown below in Table 2. The typical range of arachidonic acid and the sum of these selected fatty acids is also shown, demonstrating that the sum of these nine fatty acid residues account for 94.8-95.4% of the oil. The nine fatty acids listed in Table 2 are monitored as a quality indicator of the finished *M. alpina* oil.

Table 2. Typical Fatty Acid Composition for Hubei Fuxing *M. alpina* Oil

Fatty Acid	Hubei Fuxing ARA-rich oil (% of Fatty Acids)
Palmitic Acid (C16:0)	9.6-9.9%
Stearic Acid (C18:0)	6.3-6.6%
Oleic Acid (C18:1n-9)	5.3-5.7%
Linoleic Acid (C18:2n-6)	7.1-8.5%
gamma-Linolenic Acid (GLA) (C18:3n-6)	2.7-2.9%
Dihomo-gamma-linolenic acid (DGLA) (C20:3n-6)	5.0-5.7%
Behenic Acid (C22:0)	2.9-3.0%
Lignoceric Acid (C24:0)	9.1-9.7%
Arachidonic Acid (C20:4n-6)	44.8-46.0%
Sum of Fatty Acids: C16:0, C18:0, C18:1n-9, C18:2n-6, C18:3n-6, C20:3n-6, C22:0, C24:0, C20:4n-6	94.8-95.4%

15. *On p.34, it is noted: "all the sterols present in the subject of this GRAS Notice are ubiquitous in food, are found in the commonly used sources of fatty acids in infant formula (e.g., corn, palm, safflower, soybean, and sunflower oils), and do not pose safety concerns (Xu et al., 2018; Yang et al., 2019)." Further, desmosterol and 24-methyl desmosterol are noted as common to other M. alpina oils that have been the subject of previous GRAS notices for use in infant formula. However, it is unclear which vegetable oils contain desmosterol and 24-methyl desmosterol. Please provide additional context to the statement that the sterols present in M. alpina oil are ubiquitous in vegetable oils.*

We wish to clarify that desmosterol and 24-methyl desmosterol are not found in vegetable oils, although the other sterols present in *M. alpina* oil are also found in other food

ingredients, including the vegetable oils used to manufacture infant formula (Leman 2009; Xu et al. 2018). Desmosterol and 24-methyl desmosterol are common to other *M. alpina* oils that are GRAS for use in infant formula such as the subjects of GRNs 000041, 000080, 000326, and 000730.

Microbiology:

16. *Given that M. alpina is described in the notice as non-toxigenic and food-grade materials are used in the fermentation medium, we would not expect aflatoxin to be present in M. alpina oil. Unless aflatoxins are introduced by the fermentation medium, we suggest that Hubei Fuxing omits this specification from the notice.*

    Although aflatoxin is not expected to be present in the subject of GRN 001115, an aflatoxin specification is included to meet the regulatory requirements of other jurisdictions.

17. *In Table 9 (p.28), Hubei Fuxing indicates a limit for Cronobacter sakazakii of "not detected" in 100 mL. Please clarify the method used to detect C. sakazakii and the amount of sample in grams (e.g., 10 g) that is analyzed in accordance with the validated method.*

    The method used to detect *C. sakazakii* is performed with the amount of sample in grams and analyzed according to the compendial method described in GB 4789.40-2016 (attached).

18. *Please provide an appropriate method for the detection of C. sakazakii in oil. The method cited in the notice (GB 4789.40-2016) is for the detection of C. sakazakii in powdered infant formula.*

    The current compendial method GB 4789.40-2016 is for the examination of *Cronobacter* (*Enterobacter*) *sakazakii* in food and infant formula, milk, and dairy products and their raw materials, such as *M. alpina* oil (see Scope, page 4 of the attached method). *M. alpina* oil is an ingredient used in infant formula; therefore, GB 4789.40-2016 is an appropriate method for the detection of *C. sakazakii* in *M. alpina* oil. The current method replaced GB 4789.40-2010, which was intended for the detection of *Enterobacter sakazakii* from dehydrated powdered milk for export.

Toxicology:

19. *As noted on p.34, Shimizu et al., 1992 and Nes and Nichols, 2006 identified desmosterol as the primary sterol produced by M. alpina. However, the primary sterol in the article of commerce is 24-methyldesmosterol.[1] To support the safety of the ingredient, please provide a tabular comparison of the sterol profile of the article of commerce to an M. alpina oil previously concluded to be GRAS and/or cite a published safety study for an M. alpina oil that also has 24-methyldesmosterol as its principal sterol. ([1]Synonyms for desmosterol include: cholesta-5,24-dien-3β-ol; synonyms for 24-methyldesmosterol include: 24-methylcholesta-5,24-dien-3β-ol, ergosta-5,24-dien-3β-ol.)*

    The *M. alpina* oil that was the subject of GRN 000326 (RAO) contained 24-methyl desmosterol as the most abundant sterol (page 43, stamped page 53, GRN 326). The subjects

of GRN 000041 and 000080 and GRN 000730 also contained >10% of the sterols as 24-methyl cholesta-5,24(25)-dien-3β-ol, a synonym for 24-methyl desmosterol. It is important to note that these results were obtained using different methods and there is no compendial method validated for the detection of fungal sterols in *M. alpina* oil; therefore, it is not possible to accurately compare of the sterol profile of this GRAS Notice with those of other *M. alpina* oils that are GRAS.

Importantly, the *M. alpina* oil that was the subject of GRN 000326 was the test article used in Casterton et al. (2009). This report describes the results of a battery of genotoxicity tests and a subchronic toxicity assay in rats. RAO was not genotoxic as assessed by a reverse mutation (Ames) assay, in vitro chromosomal aberration test, and a gene mutation test up to 5000 µg/mL (5000 µg/plate in the Ames assay). The no observed adverse effect level (NOAEL) for RAO was determined to be 5% in the diet, or approximately 3170 mg/kg/day (Casterton et al. 2009).

20. *On pp.49 and 58, Hubei Fuxing describes its literature search strategy for publications that support the safety of M. alpina oil. However, we note that the searches were limited to either clinical trials in infants or reports of allergy. Please provide the results of an updated and unconstrained literature search, including search terms, dates, and databases, to support the safety of M. alpina oil in infant formula.*

Although not discussed in the Notice, a literature search was performed to identify any new toxicology or genotoxicity studies performed using *M. alpina* oil as the test article, in addition to the literature searches for clinical studies and allergenicity. The literature search was performed using PubMed and GoogleScholar on May 25, 2022. The search terms were "arachidonic acid" AND "*Mortierella alpina*" in combination with each of the following words: safety evaluation, subchronic toxicity, acute toxicity, reproductive toxicity, developmental toxicity, or genotoxicity. No new toxicology or genotoxicity studies were identified that had not been discussed in the previous *M. alpina* oil GRAS Notice that received no questions from the FDA in 2021 (GRN 000963).

Should you need any additional information, please do not hesitate to contact me at 301-775-9476 or ckruger@spherixgroup.com.

Sincerely,

Claire L. Kruger, Ph.D. D.A.B.T.
Managing Partner

Attachments:

1. March 30, 2012, FDA letter describing the suitability of the aluminum bottles for food contact use.
2. Method used to detect *C. sakazakii,* GB 4789.2016, National Standard of the People's Republic Of China

REFERENCES

Casterton, P. L., L. L. Curry, B. A.R. Lina, A. P.M. Wolterbeek, and C. L. Kruger. 2009. "90-Day Feeding and Genotoxicity Studies on a Refined Arachidonic Acid-Rich Oil." Food and Chemical Toxicology 47 (10): 2407–18. https://doi.org/10.1016/j.fct.2009.06.036.

GRN 000041, DHASCO (docosahexaenoic acid-rich single-cell oil) and ARASCO (arachidonic acid-rich single-cell oil), Martek Biosciences Corporation, 2001, https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=41.

GRN 000080, ARASCO (arachidonic acid-rich single-cell oil), Mead Johnson Nutritionals, 2001, https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=80.

GRN 000094, Docosahexaenoic acid-rich oil from tuna (DHA-rich tuna oil) and arachidonic acid-rich oil from Mortierella alpina (AA-rich fungal oil), Ross Products Division, Abbott Laboratories, 2006, https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=94.

GRN 000326, Arachidonic acid rich oil from M. alpina strain I_{49}-N_{18}, Cargill, Inc., 2011, https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=326.

GRN 000730, Arachidonic acid oil produced in Mortierella alpina, Linyi Youkang Biology Co., Ltd., 2018, https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=730.

GRN 000963. Fungal oil (≥40% arachidonic acid) from Mortierella alpina, BASF Corporation, 2021, https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=963.

Leman, J. 2009. "Lipids, Production." In Encyclopedia of Microbiology (Third Edition), edited by Moselio B T - Encyclopedia of Microbiology (Third Edition) Schaechter, 393–406. Oxford: Academic Press. https://doi.org/https://doi.org/10.1016/B978-012373944-5.00170-X.

Xu, Baocheng, Liangxiao Zhang, Fei Ma, Wen Zhang, Xiupin Wang, Qi Zhang, Denglin Luo, Hongyan Ma, and Peiwu Li. 2018. "Determination of Free Steroidal Compounds in Vegetable Oils by Comprehensive Two-Dimensional Gas Chromatography Coupled to Time-of-Flight Mass Spectrometry." Food Chemistry 245 (March 2017): 415–25. https://doi.org/10.1016/j.foodchem.2017.10.114.

Product delivered by TOURNAIRE SA: Plain aluminium bottle

APPENDIX 2: FDA Letter

DEPARTMENT OF HE. ALTH& HUMAN SERVICES — Public Health Service

Food and Drug Administration
College Park, MD 20740

March 30, 2012

Benoit Ramet
Elemental Container
(Subsidiary of Tournaire S.A.)
860 Springfield Road South
Union. NJ 07083

Dear Mr. Ramet:

This is in reply to your inquiry concerning FDA's opinion of the suitability for food contact use of two unalloyed aluminums whose compositions are described as follows (maximum percent by weight, except for aluminum):

Designation	Al (min)	Si	Fe	Cuo	Mn	Mg	Zn	Ti	Others (each)*
1070A	99.7	0.20	0.25	0.03	0.03	0.03	0.07	0.03	0.03
1050A	99.5	0.25	0.4	0.05	0.05	0.05	0.07	0.05	0.03

*The substances listed with maximum percents by weight are the prevalent impurities in these unalloyed aluminums that are routinely tested for; the percent by weight of any other trace impurity (may include Cr, Ni, Ag, B, Bi, Ga, Li, Pb, Sn, V and other metals) will be no more than 0.03[1].

Lead, cadmium and mercury may be present as unavoidable trace impurities under conditions of good manufacturing practice at levels of approximately 0.002, 0.0002, and 0.0003 percent by weight, respectively.

The aluminum will be used as bottles to ship and contain vitamins, concentrated flavors and other foods. The aluminum is expected to remain chemically inert, insoluble and resistant to corrosion and abrasion under its intended conditions of use, such that that there is little to no likelihood that its components would migrate to food at other than insignificant amounts. The aluminum will be coated as appropriate in cases wherein migration (at more than insignificant levels) of the components of the uncoated aluminum to food cannot be ruled out, such as cases involving acidic or basic conditions. FDA has historically issued favorable opinions on the use of such metals ino contact with food (assuming insignificant migration to food), and we are currently not aware of any known or likely safety problem associated with the described intended use. Therefore, assuming the conditions described above hold, we consider the aluminum acceptable for the

[1] based on "International Alloy Designations and Chemical Composition Limits for Wrought Aluminum and Wrought

intended use described and will not require premarket approval as food additives under section 409 of the Federal Food, Drug and Cosmetic Act at this time (i.e., the submission of a food contact notification, a food additive petition or a threshold of regulation exemption request will not be required).

Please do not hesitate to contact us if you have any further questions.

Sincerely.

Kenneth McAdams
Consumer Safety Officer
Division of Food Contact Notifications, HFS-275
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

GB

NATIONAL STANDARD OF

THE PEOPLE'S REPUBLIC OF CHINA

GB 4789.40-2016

National Food Safety Standard – Food Microbiological Examination – Examination of Cronobacter (Enterobacter Sakazakii)

食品安全国家标准 食品微生物学检验

克罗诺杆菌属(阪崎肠杆菌)检验

Issued on: December 23, 2016 **Implemented on: June 23, 2017**

Issued by: National Health and Family Planning Commission of the People 's Republic of China;

China Food and Drug Administration.

Table of Contents

Foreword

This Standard replaces GB 4789.40-2010 *National food safety standard Food microbiological examination: Enterobacter sakazakii*, SN/T 1632.1-2013 *Detection of enterobacter sakazakii from dehydrated powdered milk for export - Part 1: Isolation and enumeration*.

Compared with GB 4789.40-2010, the main changes in this Standard are as follows:

- modified the standard's name to "National Food Safety Standard - Food Microbiological Examination - Examination of Cronobacter (Enterobacter Sakazakii)";

- modified the number of suspicious colonies picked.

National Food Safety Standard – Food Microbiological Examination – Examination of Cronobacter (Enterobacter Sakazakii)

1 Scope

This Standard specifies the examination method for Cronobacter in food.

This Standard applies to the examination of Cronobacter in infant formula, milk and dairy products and their raw materials.

2 Equipment and materials

In addition to microbial laboratory routine sterilization and training equipment, other equipment and materials are as follows:

2.1 Thermostat incubator: 25°C ± 1°C, 36°C ± 1°C, 44°C ± 0.5°C

2.2 Refrigerator: 2°C ~ 5°C

2.3 Constant temperature water bath: 44°C ± 0.5°C

2.4 Balance: resolution of 0.1 g

2.5 Homogenizer

2.6 Oscillator

2.7 Sterile pipettes: 1 mL (with 0.01 mL scale), 10 mL (with 0.1 mL scale) or micro pipette and suction head

2.8 Sterile conical flask: capacity of 100 mL, 200 mL, 2000 mL

2.9 Sterile Petri dish: 90 m in diameter

2.10 pH meter or pH colorimetric tube or precision pH test paper

2.11 Automatic microbial biochemical identification system

3 Medium and reagent

3.1 Buffer peptone water: see A.1

3.2 Modified lauryl sulfate tryptose broth-vancomycin medium, mLST-Vm): see A.2

3.3 Enterobacter sakazakii chromogenic medium

3.4 Trypticase soy agar, TSA: see A.3

3.5 Biochemical identification kit

3.6 Oxidase reagent: see A.4

3.7 L-lysine decarboxylase medium: see A.5

3.8 L-ornithine decarboxylase medium: see A.6

3.9 L-arginine bishydrolase medium: see A.7

3.10 Carbohydrate fermentation medium: see A.8

3.11 Citrus citrate medium: see A.9

Method One Qualitative test of Cronobacter

4 Examination procedures

See Figure a for the examination procedures of Cronobacter.

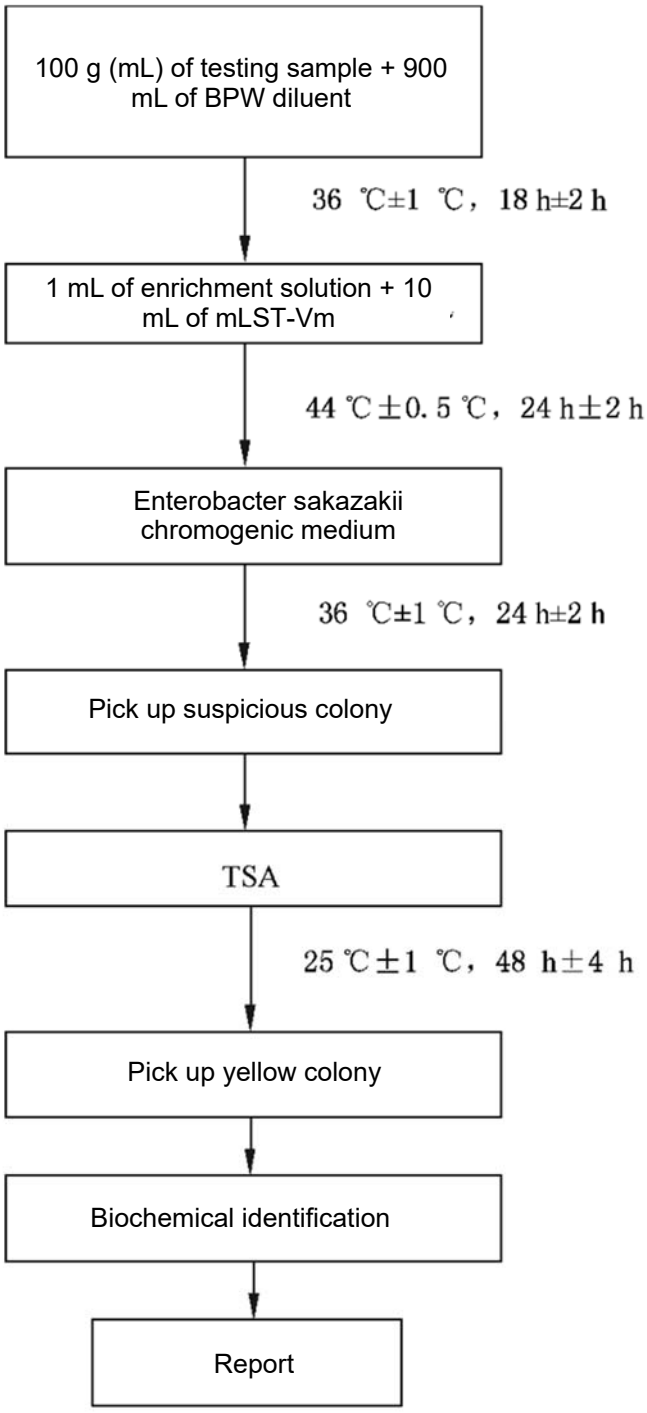


Figure 1 -- Examination procedures of Cronobacter

5 Operation steps

5.1 Pre-enrichment and enrichment

Take 100 g (mL) of testing sample into a sterile conical flask. Add into 900 mL of buffered peptone water that has been preheated to 44°C. Shake slowly to full dissolution by hand, and incubate at 36°C ± 1°C for 18 h ± 2 h. Transfer 1

mL to 10 mL mLSt-Vm broth, incubate at 44°C ± 0.5°C for 24 h ± 2 h.

5.2 Separation

5.2.1 Mix gently mLST-Vm broth culture. Respectively take 1 ring of the culture and inoculate in two platelets of Enterobacter sakazakii. The color medium shall meet the requirements of GB 4789.28. Culture at 36°C ± 1°C for 24h ± 2h, or according to the requirements for culture medium.

5.2.2 Pick up at least 5 suspicious colonies. When it is less than 5, pick all suspicious colonies to inoculate in TSA plate. Culture at 25°C ±1°C for 48 h ± 4 h.

5.3 Identification

Directly pick yellow suspicious colonies from TSA plate for biochemical identification. The main biochemical characteristics of Cronobacter are shown in Table 1. It can select biochemical identification kit or automatic microbial biochemical identification system.

Table 1 -- Main biochemical characteristics of Cronobacter

Biochemical test		Characteristics
Generation of yellow pigment		+
Oxidase		-
L-lysine decarboxylase		-
L-ornithine decarboxylase		(+)
L-arginine bishydrolase		+
Citric acid hydrolysis		(+)
Fermenting	D-sorbitol	(-)
	L-rhamnose	+
	D-sucrose	+
	D-melibiose	+
	Amygdalin	+
NOTE: +> 99% positive; -> 99% negative; (+) 90% to 99% positive; (-) 90% to 99% negative.		

6 Result and report

Comprehensive colony morphology and biochemical characteristics shall be reported as Cronobacter detected or not per 100 g (mL) of sample.

Method Two Counting of Cronobacter

7 Operation steps

7.1 Sample dilution

7.1.1 Solid and semi-solid samples: sterilely weigh 100 g, 10 g, 1g of samples, respectively add into 900 mL, 90 mL, 9 mL of BPW that have been preheated to 44°C. Gently shake to fully dissolved, make 1:10 sample homogenizing solution, culture at 36°C ± 1°C for 18h ± 2h. Respectively transfer 1 mL to inoculate in 10 mL LmST-Vm broth, culture at 44°C ± 0.5°C for 24h ± 2h.

7.1.2 Liquid sample: use a sterile pipette to take 100 mL, 10 mL, 1 mL of samples, respectively add into 900 mL, 90 mL, 9 mL of BPW that have been preheated to 44°C. Gently shake to fully dissolved, make 1:10 sample homogenizing solution, culture at 36°C ± 1°C for 18h ± 2h. Respectively transfer 1 mL to inoculate in 10 mL LmST-Vm broth, culture at 44°C ± 0.5°C for 24h ± 2h.

7.2 Separation, identification

Same with 5.2 and 5.3.

8 Result and report

Combining the colony morphology, biochemical characteristics and according to the confirmed number of positive tubes of Cronobacter, check MPN search table and report the MPN value of Cronobacter per 100 g (mL) of sample (see Table B.1).

Annex A

Medium and reagent

A.1 Buffer peptone water (BPW)

A.1.1 Composition

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$)	9.0 g
Potassium dihydrogen phosphate	1.5 g
Distilled water	1000 mL

A.1.2 Preparation method

Heating and stirring to dissolved, adjust pH to 7.2 ± 0.2, autoclave at 121°C for 15 min.

A.2 Modified lauryl sulfate tryptose broth-vancomycin medium, mLST-Vm

A.2.1 Modified lauryl sulfate tryptone (mLST) broth

A.2.1.1 Composition

Sodium chloride	34.0 g
Tryptone	20.0 g
Lactose	5.0 g
Potassium dihydrogen phosphate	2.75 g
Dipotassium phosphate	2.75 g
Sodium dodecyl sulfate	0.1 g
Distilled water	1000 mL

A.2.1.2 Preparation method

Heating and stirring to dissolved, adjust pH to 6.8 ± 0.2. Sub-packaging, 10 mL for each tube, autoclave at 121°C for 15 min.

A.2.2 Vancomycin solution

A.2.2.1 Composition

Vancomycin	10.0 mg
Distilled water	10.0 mL

A.2.2.2 Preparation method

Dissolve 10.0 mg of vancomycin in 1.00 mL of distilled water, filter for

sterilization. The vancomycin solution can be stored at 0°C ~ 5°C for 15 d.

A.2.3 Modified lauryl sulfate tryptose broth - vancomycin medium, mLST-Vm

Add 0.1 mL of vancomycin solution into per 10 mL of mLST. The final concentration of vancomycin in the mixture is 10 μg/mL.

NOTE: mLST-Vm must be used within 24h.

A.3 Tryptone soy agar (T S A)

A.3.1 Composition

Tryptone	15.0 g
Plant peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1000 mL

A.3.2 Preparation method

Heating and stirring to dissolved. Boiling for 1 min. Adjust pH to 7.3 ± 0.2. Autoclave at 121°C for 15 min.

A.4 Oxidase reagent

A.4.1 Composition

N, N, N ', N' - tetramethylphenylene diamine hydrochloride	1.0 g
Distilled water	1000 mL

A.4.2 Preparation method

Make a small amount of fresh preparation. Store in the refrigerator from light. Use within 7d.

A.4.3 Test method

Use a glass rod or a disposable inoculation needle to pick up a single characteristic colony and coat it on a filter paper plate moistened with an oxidase reagent. If the filter paper does not become magenta, purple or dark blue within 10s, the oxidase test is negative, otherwise the oxidase test is positive.

NOTE: Do not use nickel/chromium material in the experiment.

A.5 L-lysine decarboxylase medium

A.5.1 Composition

L-lysine monohydrochloride	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Bromocresol purple	0.015 g
Distilled water	1000 mL

A.5.2 Preparation method

Heat and dissolve the components, and if necessary, adjust pH to 6.8 ± 0.2. Sub-packaging 5 mL for each tube. Autoclave at 121°C for 15 min.

A.5.3 Experimental method

Inoculate the culture into the L-lysine decarboxylase medium, just under the liquid level of the liquid medium. Culture at 30°C ± 1°C for 24h ± 2h. Observe the results. L-lysine decarboxylase test is positive; the medium is purple; the negative is yellow; blank control tube is purple.

A.6 L-ornithine decarboxylase medium

A.6.1 Composition

L-ornithine monohydrochloride	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Bromocresol purple	0.015 g
Distilled water	1000 mL

A.6.2 Preparation method

Heat and dissolve the components, and if necessary, adjust pH to 6.8 ± 0.2. Sub-packaging 5 mL for each tube. Autoclave at 121°C for 15 min.

A.6.3 Experimental method

Inoculate the culture into the L-ornithine decarboxylase medium, just under the liquid level of the liquid medium. Culture at 30°C ± 1°C for 24h ± 2h. Observe the results. L-ornithine decarboxylase test is positive; the medium is purple; the negative is yellow.

A.7 L-arginine bishydrolase medium

A.7.1 Composition

L-arginine monohydrochloride	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Bromocresol purple	0.015 g
Distilled water	1000 mL

A.7.2 Preparation method

Heat and dissolve the components, and if necessary, adjust pH to 6.8 ± 0.2. Sub-packaging 5 mL for each tube. Autoclave at 121°C for 15 min.

A.7.3 Experimental method

Inoculate the culture into the L-arginine decarboxylase medium, just under the liquid level of the liquid medium. Culture at 30°C ± 1°C for 24h ± 2h. Observe the results. L-arginine decarboxylase test is positive; the medium is purple; the negative is yellow.

A.8 Carbohydrate fermentation medium

A.8.1 Basal medium

A.8.1.1 Composition

Casein (enzyme digestion)	10.0 g
Sodium chloride	5.0 g
Phenol red	0.02 g
Distilled water	1000 mL

A.8.1.2 Preparation method

Heat and dissolve the components, and if necessary, adjust pH to 6.8 ± 0.2. Sub-packaging 5 mL for each tube. Autoclave at 121°C for 15 min.

A.8.2 Sugar solution (D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin)

A.8.2.1 Composition

Sugar	8.0 g
Distilled water	100 mL

A.8.2.2 Preparation method

Respectively weigh 8 g of D-sorbitol, L-rhamnose, D-sucrose, D-honey disaccharide, amygdalin and dissolve into 100 mL of distilled water. Filter for sterilization, make 80 mg/mL sugar solution.

A.8.3 Complete medium

A.8.3.1 Composition

Basal medium	875 mL
Sugar solution	125 mL

A.8.3.2 Preparation method

Aseptically add each saccharide solution to the basal medium and mix well.

Sub-package into sterile test tubes, 10 mL per tube.

A.8.4 Experimental method

Inoculate the culture into the L-arginine decarboxylase medium, just under the liquid level of the liquid medium. Culture at 30°C ± 1°C for 24h ± 2h. Observe the results. Carbohydrate fermentation test is positive. The medium is yellow. The negative is red.

A.9 Citrus citrate medium

A.9.1 Composition

Sodium citrate	2.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	1.0 g
Ammonium dihydrogen phosphate	1.0 g
Magnesium sulfate	0.2 g
Bromothymol blue	0.08 g
Agar	8.0g ~ 18.0g
Distilled water	1000 mL

A.9.2 Preparation method

Heat and dissolve the components, and if necessary, adjust pH to 6.8 ± 0.2. Sub-packaging 5 mL for each tube. Autoclave at 121°C for 15 min to make bevel.

A.9.3 Experimental method

Inoculate the culture into the entire medium slope. Culture at 36°C ± 1°C for 24h ± 2h. Observe the results. Positive medium becomes blue.

Annex B

The most probable number (MPN) search table for Cronobacter

The most probable number (MPN) search table for Cronobacter per 100 g(mL) of testing sample is shown in Table B.1.

The number of positive tubes

95% confidence limit

Table B.1 -- The most probable number (MPN) search table

			MPN						MPN		
100	10	1		Lower	Upper	100	10	1		Lower	Upper
0	0	0	<0.3	—	0.95	2	2	0	2.1	0.45	4.2
0	0	1	0.3	0.015	0.96	2	2	1	2.8	0.87	9.4
0	1	0	0.3	0.015	1.1	2	2	2	3.5	0.87	9.4
0	1	1	0.61	0.12	1.8	2	3	0	2.9	0.87	9.4
0	2	0	0.62	0.12	1.8	2	3	1	3.6	0.87	9.4
0	3	0	0.94	0.36	3.8	3	0	0	2.3	0.46	9.4
1	0	0	0.36	0.017	1.8	3	0	1	3.8	0.87	11
1	0	1	0.72	0.13	1.8	3	0	2	6.4	1.7	18
1	0	2	1.1	0.36	3.8	3	1	0	4.3	0.9	18
1	1	0	0.74	0.13	2	3	1	1	7.5	1.7	20
1	1	1	1.1	0.36	3.8	3	1	2	12	3.7	42
1	2	0	1.1	0.36	4.2	3	1	3	16	4	42
1	2	1	1.5	0.45	4.2	3	2	0	9.3	1.8	42
1	3	0	1.6	0.45	4.2	3	2	1	15	3.7	42
2	0	0	0.92	0.14	3.8	3	2	2	21	4	43
2	0	1	1.4	0.36	4.2	3	2	3	29	9	100
2	0	2	2	0.45	4.2	3	3	0	24	4.2	100
2	1	0	1.5	0.37	4.2	3	3	1	46	9	200
2	1	1	2	0.45	4.2	3	3	2	110	18	410
2	1	2	2.7	0.87	9.4	3	3	3	>110	42	—

NOTE 1: This sample uses three samples [100 g(mL), 10 g(mL) and 1 g(mL)]. Each sample is inoculated with 3 tubes.
NOTE 2: If the amount of sample listed in the table uses 1000 g(mL), 100 g(mL) and 10 g(mL), the number in the table shall be reduced by 10 times. If it uses 10 g(mL), 1 g(mL) and 0.1 g(mL), the number in the table shall be increased by 10 times, and similarly as to the others.

END

Twelve pages have been removed in accordance with copyright laws. The removed reference citation is:

P.L. Casterton, L.L. Curry, B.A.R. Lina, A.P.M Wolterbeek, C.L. Kruger, " 90-Day feeding and genotoxicity studies on a refined arachidonic acid-rich oil", Food and Chemical Toxicology, vol. 47, pp. 2407-2418, 2009.

Eleven pages have been removed in accordance with copyright laws. The removed reference citation is:

B. Xu, L. Zhang, F. Ma, et al., " Determination of free steroidal compounds in vegetable oils by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry", Food Chemistry, vol. 245, pp. 415-425, 2018.

Fourteen pages have been removed in accordance with copyright laws. The removed reference citation is:

J. Leman, “Lipids, Production”, Applied Microbiology: Industrial, pp. 393-406, 2009.