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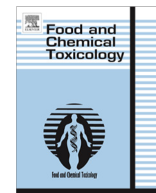

Safety evaluation of oleic-rich triglyceride oil produced by a heterotrophic microalgal fermentation process

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ABSTRACT

Numbers of macro- and microalgae have been used as food sources in various cultures for centuries. Several microalgae are currently being developed as modern food ingredients. The dietary safety of oleic-rich microalgal oil produced using a heterotrophic fermentation process was assessed in a 13-week feeding trial in rats with genotoxic potential evaluated using *in vitro* and *in vivo* assays. In the genotoxicity assays, the test oil was not mutagenic in *Salmonella typhimurium* or *Escherichia coli* tester strains (⩽5000 μg/plate) with or without metabolic activation. Further, no clastogenic response occurred in chromosome aberration assays in the bone marrow of mice administered a single intraperitoneal dose (2000 mg/kg). In the subchronic study, rats consumed feed containing 0, 25,000, 50,000 or 100,000 ppm oleic-rich oil for 90 days. No treatment-related mortalities or adverse effects occurred in general condition, body weight, food consumption, ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights or histopathology. Although several endpoints exhibited statistically significant effects, none were dose-related or considered adverse. Taking all studies into consideration, the NOAEL for the oleic-rich oil was 100,000 ppm, the highest concentration tested and equivalent to dietary NOAELs of 5200 mg/kg bw/day and 6419 mg/kg bw/day in male and female rats, respectively.

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1. Introduction

New ingredient oils that are high in monounsaturated fatty acids such as oleic acid (C18:1 *cis n*-9) are actively being sought by the food industry for use in prepared food items and as fry oils. Health concerns are driving a reduction of saturated fats and removal of trans-fats (i.e., partially hydrogenated oils) from many foods in the American diet. In response, a high demand has been created for replacement oils that have the ability to retain consistent flavor profiles, provide the same or even improved shelf-life stability, and potentially impart health benefits. As a means of creating alternatives to the unhealthy but stable trans-fats, sunflower and canola plants have recently been bred to produce mid- and high-oleic oils. In addition to these more traditional oil sources, technological advances have made large-scale heterotrophic fermentation possible for the generation of algal-derived oils. Microalgae are grown in the absence of light in fermentation tanks and use sugars as their energy source. The new oleic-rich microalgal oils that are produced using this technology are now being evaluated and developed as food ingredients. Regarding health benefits, the oleic-rich microalgal oils are expected to provide the same potential benefits as the other high oleic oils, olive oil, canola oil (traditional and high-oleic), sunflower oil (traditional and high-oleic), and peanut oil. Clinical studies have shown that consumption of these oils decreases total and LDL cholesterol and triglycerides (without lowering HDL cholesterol), decreases C-reactive protein, and attenuates the activity of coagulant factors, thereby potentially providing a reduced risk of cardiovascular disease, ischemic heart disease, and stroke (Allman-Farinelli et al., 2005; Jenkins et al., 2010; Kris-Etherton et al., 1999; Larsen et al., 1999).

Abbreviations: 2-AA, 2-aminoanthracene; AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care International; ANOVA, Analysis of Variance; AOAC, Association of Analytical Communities; AOCS, American Oil Chemists' Society; ASTM, American Society for Testing and Materials; bw, body weight; CPA, cyclophosphamide; cps, centipoise; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; g, gram; GLP, good laboratory practice; GRAS, Generally Recognized as Safe; GRN, GRAS Notification; HDL, high-density lipoprotein; i.p., intraperitoneal; ISO, International Organization for Standardization; IUPAC, International Union of Pure and Applied Chemistry; kg, kilogram; LDL, low-density lipoprotein; mg, milligram; MMS, methylmethanesulfonate; MTD, maximum tolerated dose; ppm, parts-per-million; 4-NOPD, 4-nitro-*o*-phenylene-diamine; NOAEL, no-observed-adverse-effect level; OECD, Organisation for Economic Co-operation and Development; OSI, Oil Stability Index; OSD, OpenSource Diet; TFA, total fatty acid; US FDA, United States Food and Drug Administration.

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Prototheca moriformis is an achlorophyllous microalga found ubiquitously in the environment that naturally produces large quantities of triglyceride oil and is related to *Chlorella protothecoides* (also called *Auxenochlorella protothecoides*; Huss et al., 1999). Solazyme, Inc., (South San Francisco, CA) uses this alga, which was engineered, for the production of oleic acid (C18:1). The resulting algae are stable microorganisms that produce a clear, pale to wheat yellow oleic-rich oil composed primarily of triglycerides (>98%) with small amounts of diglycerides (~1.5%), and trace amounts of monoglycerides (<0.01%). Oleic acid, a long-chain omega-9 fatty acid, can be present in concentrations of >85%, of which <0.1% is as the free fatty acid. No erucic acid was detected.

The US Food and Drug Administration (FDA) has accepted with "no questions" several Generally Recognized as Safe (GRAS) Notifications (GRNs) for microalgal-derived ingredients including a high-DHA oil (35–45% docosahexaenoic acid by weight) extracted from *Schizochytrium* sp.,[1] a high-DHA oil (~45% DHA by weight) extracted from *Ulkenia* sp. SAM2179,[2] oil extracted from *C. protothecoides*,[3] and a high-lipid algal flour[4] produced from *C. protothecoides*. Prior to bringing a new microalgal-derived food onto the market for human consumption, the safety of the ingredient must first be demonstrated. To this end, the dietary safety of oleic-rich microalgal oil produced using a heterotrophic fermentation process was assessed in a comprehensive 13-week dietary study in rats and two genotoxicity assays, an *in vitro* mutagenicity assay (the bacterial reverse mutation assay) and an *in vivo* clastogenicity assay (the chromosome aberration assay).

2. Materials and methods

2.1. Test substance and diet preparation

The oleic-rich algal oil (Lot # RBD540, provided by Solazyme, Inc., South San Francisco, CA) was a clear, pale to wheat yellow, refined, bleached and deodorized oil isolated from an engineered strain of *P. moriformis*. Under current Good Manufacturing Practice, the oil was prepared via fed-batch axenic fermentation of microalgae. Temperature, pH, agitation, aeration, and additions of glucose and nutrients were controlled throughout. After fermentation, the culture broth was inactivated by drying. The crude oil was then extracted from the dried biomass and processed using standard edible oil refining steps, including clarification, degumming, bleaching, and deodorization.

Physical and chemical characteristics and composition of the test oil (Table 1), in addition to potential contaminants, were determined using AOCS, ASTM, IUPAC and internal methods. The oil was composed of neutral-flavored oil[5] (approximately 100%), which was almost exclusively in the form of triglycerides (98.6%) with lesser amounts of diglycerides (1.58%) and no detectable monoglycerides. Free fatty acids (0.019%) and polar compounds (1.63%) were also present. Non-saponifiable matter comprised 0.65% of the oil and moisture was determined to be 352 ppm. Oleic acid was the predominant fatty acid comprising the oil (88.50 Area% of TFA) followed by palmitic and stearic acids (7.30 and 1.06 Area% of TFA, respectively) and lesser amounts of other fatty acids. No erucic acid was detected. Minerals (including heavy metals), tocopherols and sterols were also quantified in the test oil (Table 1).

Pheophorbide A is a naturally occurring degradation product of chlorophyll associated with photosensitive dermatitis in humans (Jassby, 1988; Jitsukawa et al., 1984). The oil was analyzed for this chlorophyll-related pigment by high-performance liquid chromatography with fluorescence detection[6] at UBE Analytical Laboratories (Fullerton, CA). Assays for algal toxins and cyanotoxins included amnesic shellfish poisoning toxins (domoic acid), diarrhetic shellfish poisoning toxins (okadaic acid, dinophysistoxin-1, pectenotoxin-2, azaspiracid-1, yessotoxin, and hommo-yessotoxin), paralytic shellfish poisoning toxins (gonyautoxins 1–6; decarbamoylgonyautoxins 2 and 3; saxitoxin; decarbamoylsaxitoxin; neosaxitoxin and ciguatoxins 1–4), cyanobacterial toxins (microcystin-RR, -YR, -LR, -LW, -LF, -LA, -WR, -LY and -HtyR and dm-microcystin-RR and -LR), nodularin, anatoxin and cylindrospermopsin. All algal and cyanotoxin assays were conducted by liquid chromatography with tandem mass spectrometric detection[7] at Food GmbH Analytik & Consulting (Jena, Germany).

[1] GRN 137 "Algal oil (*Schizochytrium* sp.)" (US FDA, 2004).
[2] GRN 319 "Micro-algal oil *Ulkenia* sp. SAM2179" (US FDA, 2010).
[3] GRN 384 "Algal oil derived from *Chlorella protothecoides* strain S106 (Cp algal oil)" (US FDA, 2012).
[4] GRN 469 "*Chlorella protothecoides* strain S106 flour with 40-70% lipid (algal flour)" (US FDA, 2013).
[5] An oil which does not impart its own flavor to foods.
[6] Limits of detection were 0.5 ppm for pheophorbide A.
[7] Limits of detection ranged from 0.0008–0.1 μg/g in the oil.

Research Diets, Inc. (New Brunswick, NJ) prepared the test diets for the 13-week feeding trial in rodents from modified OpenSource Diets (OSDs) formulated to contain sufficient oleic-rich algal oil (with soybean oil as the balance) to achieve target concentrations 0 ppm (0.0%), 25,000 ppm (2.5%), 50,000 ppm (5.0%) and 100,000 ppm (10.0%) and to ensure equivalent fat and energy content across dose groups (Table 2). All diets were stored under refrigerated conditions. The oleic-rich oil was stored at room temperature (15–25 °C). The stability, homogeneity and concentration of test substance in each test diet were evaluated via analysis of the test oil in collected feed samples, with samples being frozen until assayed. For the *in vitro* bacterial reverse mutation assays, the test oil was suspended in distilled water and processed by ultrasound for ten minutes at 37 °C just prior to dilution. For the *in vivo* chromosome aberration assay, the test oil was diluted in cottonseed oil.

2.2. Chemicals and materials

Materials, including 2-aminoanthracene (2-AA), cottonseed oil, cyclophosphamide (CPA), methylmethanesulfonate (MMS), physiological saline (0.9% sodium chloride), and sodium azide (NaN_3) were purchased from Sigma–Aldrich Corp. (St. Louis, MO); 4-nitro-*o*-phenylene-diamine (4-NOPD) was purchased from Fluka Analytical (Sigma–Aldrich). The S9 metabolic activation mix was prepared from the supernatant fraction (0.5 parts) of rat liver homogenate (from livers of male Wistar rats induced for three consecutive days by oral exposure to 80 mg/kg bw phenobarbital and 100 mg/kg bw β-naphthoflavone) and cofactor solution (9.5 parts). Nutrient Broth No. 2 (for *Salmonella typhimurium* test strains) was obtained from Oxoid Ltd. (Basingstoke, Hampshire, England) with Luria Bertani medium (for the *Escherichia coli* strain) containing tryptone and yeast extract from Roth GmbH (Karlsruhe, Germany) and Oxoid Ltd. Distilled water (Lot Nos. 120711 and 121022) for the mutagenicity assay was provided by BSL Bioservice (Planegg, Germany). Distilled in-house water was used, unless otherwise specified.

2.3. Animals and organisms

For the 13-week subchronic dietary study conducted at Product Safety Labs (Dayton, NJ), CRL Sprague–Dawley (SD) CD® IGS rats (male and female) were obtained from Charles River Laboratories (Kingston, NY). In-house veterinarian staff visually inspected all rats at delivery and during a 6-d acclimation. At study initiation, the rats were 7–8 weeks old. Body weight variations remained within ±20% of the measured mean for both sexes (±2.6% for males with 203.2 ± 5.3 g mean body weight and 194–215 g range; ±4.8% for females with 164.5 ± 7.9 g mean body weight and 149–179 g range). Rats were caged individually under environmentally-controlled conditions including photoperiods (12-h light:dark), air exchanges (⩾10 times per h), room temperatures (19–23 °C) and humidity (45–67%). Filtered tap water and the customized test diets were provided *ad libitum*.

Tester strains used in the bacterial reverse mutation assays performed at BSL Bioservice were obtained from Molecular Toxicology Inc. (Boone, NC) (*S. typhimurium* TA 98 and TA1535 and *E. coli* WP2 *uvrA*) and Xenometrix AG (Allschwil, Switzerland) (*S. typhimurium* TA 100 and TA1537).

Male and female NMRI mice used in the *in vivo* chromosome aberration assay performed at BSL Bioservice were obtained from Charles River GmbH (Sulzfield, Germany). In-house veterinarian staff visually inspected all mice at delivery and during the acclimation period (minimum of 5 days). At dosing, the mice were 7–13 weeks old. Initial body weights ranged from 27.8 to 33.2 g (mean, 30.3 ± 1.2 g) for males and 22.7 to 26.7 g (mean, 24.9 ± 0.9 g) for females, with variations of ±8.9% and ±8.0%, respectively. Mice were housed by sex (5 animals of the same sex per cage) in animal rooms that included environmentally-controlled photoperiods (12-h), air exchanges (⩾10 times per h), room temperatures (22 ± 3 °C) and humidity (55 ± 10%). Tap water (sulfur acidified to ~pH 2.8) and Altromin 1324 maintenance diet (Altromin Spezialfutter GmbH & Co.) were provided *ad libitum*.

2.4. Guidelines

The 13-week dietary study in rats was conducted in conformance with:

- The protocol reviewed by the PSL Institutional Animal Care and Use Committee (AAALAC Accredited Unit No. 000939).
- Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 408): Health Effects, Repeated Dose 90-Day Oral Toxicity Study in Rodents (1998).

and in compliance with the following GLP regulations:

- OECD Principles of Good Laboratory Practice (as revised in 1997) in ENV/MC/CHEM (98)17, OECD, Paris (1998).
- US FDA GLP: 21 CFR 58 (1987).

The bacterial reverse mutation assay was performed in conformance with:

- Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted July 21, 1997.

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