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Genotoxicity evaluation of a fish oil concentrate containing Very Long Chain Fatty Acids

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ABSTRACT

Very long chain fatty acids (VLCFAs) are lipids found in fish with a chain length longer than C22. They represent a minor lipid fraction composing of less than 1% of the total lipid. EPAX® EVOLVE 05 is a concentrate of VLCFAs providing roughly 10 times the amount found in fish. Here we report genotoxocity studies performed in cell culture and using a rat model. No genotoxicity was noted in a bacterial reverse mutation test (AMES test). An in vitro micronucleus assay was negative with a 4-hr test item incubation but a 24-hr incubation resulted in a positive signal. This prompted further study using an in vivo Sprague Dawley rat model. Test item exposure was demonstrated by plasma measurements from Sprague Dawley rats with peak absorption at 2–4 h post administration, as expected for fatty acids. The micronucleus assay showed no genotoxicity for fish oil containing VLCFAs. Together, the data shows that VLCFAs up to the test dose of 1200 mg/kg b.w. do not show genotoxicity.

1. Introduction

Fish oil supplements are mainly focussed on the 20 and 22 carbon polyunsaturated eicosapentaenoic acid (C20, EPA) and docosahexaenoic (C22, DHA) fatty acids. There is a wealth of information describing their beneficial health effects in cardiovascular health [4], as anti-inflammatory agents [10], brain health [24] and in pregnancy [5] supporting widespread use of fish oils as supplements. Less well researched are the extension products of EPA and DHA, the so-called Very Long Chain Fatty Acids (VLCFA) which are derived by elongation and desaturation, mostly of EPA, but also DHA. These VLCFAs are products of elongation of EPA by a family of proteins called Elongation of Very Long chain fatty acids (ELOVL) and in particular ELOVL4 which is found in the brain, retina, skin and testes [14]. Knock-out of the gene coding for the ELOVL4 protein results, amongst other things, in poor skin barrier function and blindness [1,26]. Humans with a homozygous ELOVL4 mutation show severe neurological disorders, scaly skin, lack of teeth and die during childhood [2,28,9] whilst heterozygous mutation is responsible for juvenile blindness known as Stargardt 3 [30].

Fish are a source of VLCFAs with approximately 0.5% of lipid comprised of VLCFA, dependent on the species. Epax Norway AS is a manufacturer of omega-3 concentrates for use as commercial supplements for human consumption and has made an approximate 10-times VLCFA concentrate known as EPAX EVOLVE 05 using traditional manufacturing methods for concentration of marine EPA and DHA [6]. Studies in rats show that dietary intake of VLCFA results in increased levels in target tissue, supporting the role of VLCFA as a supplement to boost endogenous levels of these fatty acids [25]. Moreover, oral supplementation with synthetic VLCFA can rescue visual acuity in an animal model of blindness called Stargardt 3 [11] showing that VLCFAs given as a supplement can be utilised by target tissues and likely undergo processing to the particular active form required by the target tissue (for example a phospholipid form in the retina).

Although fish intake in modern times in most Western countries is low [19], many countries have a history of high intake of fish (for example Norway, UK and Ireland). Fish and fish oils are traditional food items around the world and are considered safe and beneficial to health ('Scientific opinion on the Tolerable upper intake level of EPA, DHA and DPA' 2012). The genotoxic potential of EPA and DHA has been assessed in a number of studies, mostly from algal preparations, without any indication that these polyunsaturated fatty acids are genotoxic [12,13,16,17,21,8]. The polyunsaturated fatty acids EPA and DHA are

Abbreviations: b.w., body weight; DHA, Docosahexaenoic acid; DMSO, Dimethylsulphoxide; ELOVL, Elongation of Very Long Chain Fatty Acids; EPA, Eicosapentaenoic acid; MN, Micronuclei; MUFA, Monounsaturated fatty acids; NOAEL, No Observable Adverse Effect Level; PCE, Polychromatic erythrocytes; PUFA, Polyunsaturated fatty acids; VLCFA, Very long chain fatty acids; VLCPUFA, Very long chain poly-unsaturated fatty acids.

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Table 1
Test product composition in genotoxicity studies.

Batch ID	Total VLCFA content (mg/g)	VLCPUFA (mg/g)	Study
EPAX EVOLVE 5 Batch: A21–664	158	55	AMES test *In vitro* micronucleus study *In vivo* micronucleus study for dose 600 mg/kg b.w.
EPAX High concentrate VLCBatch: A21–659	312	133	*In vivo* micronucleus study for doses 900 and 1200 mg/kg b.w. Target tissue study with dose 1200 mg/kg b.w.

considered safe for chronic use by the FDA at a dose level up to 3 g per day [23] and up to 5 g per day by the European food safety authority, EFSA [22].

In anchovies (fish typically used for commercial harvesting of omega-3 EPA and DHA), the VLCFA content is approximately 0.5% of total lipids. EPAX EVOLVE 05 represents approximately a 10-fold concentration. Analysis of the VLCFA content of mackerel and herring fillets shows that 2 g of EPAX EVOLVE 05 (this being the proposed intake) is equivalent to eating 90–135 g of herring fish (data not shown) which is considered a normal sized fish meal. Therefore, we consider the risk of toxicity from fish derived oils to be low due to lack of reported toxic effects of fish consumption outside of those reported for environmental toxins.

The average daily intake of fish in Norway was estimated as 45 g [3], thus average data suggests reasonable chronic intake of VLCFA, but at lower levels than those suggested for EPAX EVOLVE 05. Therefore we have performed a series of toxicological evaluations to determine the safety of VLCFA. Here, we report a series of studies to determine the genotoxic profile of VLCFAs.

2. Materials and methods

2.1. Test item

The test item (EPAX EVOLVE 05) is a marine fatty acid concentrate containing very long chain fatty acids, defined as fatty acids with a carbon chain longer than C22. The manufacture of the oil follows a quality standard required for commercial production of a fish oil concentrate for human consumption. Fatty acids in natural fish oils are mostly present as triglycerides. In order to concentrate the oils, they are converted into ethyl esters, distilled and re-converted to triglycerides. This is a common industrial process for high concentrate omega-3 fatty acids used as supplements [27].

The test item was produced in two batches for the performance of genotoxicity tests.

The composition of EPAX EVOLVE 05 batch A21–664 is provided in Supplementary Table S1. The method for analysing VLCFAs was a modified version of European Pharmacopeia method 2.4.29 “Composition of fatty acids in oils rich in omega-3-acids”.

A high concentrate VLCFA oil was made for experimental practical reasons to limit the volume of oil animals would receive during the study. In these concentrates, the overall VLCFA was increased as described in Table 1, with the increase being higher for VLCPUFA than VLCMUFA.

2.2. Genotoxicity studies

2.2.1. Bacterial reverse mutation test (AMES test)

In vitro bacterial mutagenicity assays were performed to assess the potential mutagenic effect of EPAX EVOLVE 05. The study was performed at Eurofins BioPharma, Germany in accordance with the OECD 471 guideline and performed under Good Laboratory Practice.

Reverse mutation assays were performed using *S. typhimurium* and *E. coli*. The *S. typhimurium* strains were constructed to differentiate between base pair (TA100, TA1535) and frameshift (TA98, TA1537) mutations [29] and the *E. coli* strain (WP2 uvrA) detected base substitution mutagens.

The test item was supplied as a triglyceride oil and diluted in a DMSO vehicle at approximately 8% v/v.

The toxicity of the test item was determined with tester strains TA98 and TA100 in a pre-experiment with and without metabolic activation using 3 plates for each concentration. Eight concentrations were tested, 0.0316, 0.100, 0.316, 1.0, 2.5 and 5.0 μL/plate, for toxicity and mutation induction. The experimental conditions in this pre-experiment were the same as described below for the main experiment I (plate incorporation test).

Each tester strain was cultured for 12 hrs at 37 °C in a suitable growth medium to yield 10^9 cells/mL. For the plate incorporation method, a mix of 100 μL test solution (with test item, negative or positive control), 500 μL S9 mix or S9 buffer, and 100 μL bacterial suspension. This was overlaid with 2 mL of agar. After solidification the plates were inverted and incubated at 37 °C for at least 48 h in the dark. Colonies were counted using a ProtoCOL counter (Meintrup DWS Laborgeräte GmbH). Tester strains with a low spontaneous mutation frequency like TA1535 and TA1537 were counted manually.

For the pre-incubation method. 100 μL of the test item-preparation was pre-incubated with the tester strains (100 μL) and sterile buffer or the metabolic activation system (500 μL) for 60 min at 37 °C prior to adding the overlay agar (2000 μL) and pouring onto the surface of a minimal agar plate. For each method the experiment was performed with triplicate plates.

2.2.2. In vitro mammalian micronucleus test

The in vitro micronucleus assay detects the clastogenic (causing DNA strand breakage) and aneugenic (causing abnormal chromosome number (aneuploidy)) activity in cells that have undergone cell division during or after exposure to the test item. The in vitro mammalian micronucleus study was performed at Eurofins BioPharma, Germany in accordance with the OECD 487 guideline and performed under Good Laboratory Practice. Micronuclei are signs of DNA damage but are only seen if the cell is dividing. The study therefore used exponentially growing Chinese hamster V79 cells (Eurofins, Munich). 5×10^5 cells were seeded from a frozen stock in 75 cm^2 plastic flasks with 15 mL of MEM with 10% foetal bovine serum. Metabolic activation was performed by supplementation of culture medium with an S9 liver microsomal preparation.

A pre-test was performed to determine toxicity of the test item using doses at 7.8–5000 μg/mL. For the main experiment I, 50,000 V79 cells were seeded into 25 cm^2 culture flasks. After an attachment period of 48 hrs the medium was removed, and fresh medium added containing the appropriate dose of test item or control. Test item doses ranged from 50 to 200 μg/mL. Cells were incubated for 4 hrs with or without metabolic activation, washed and incubated for a further 20 hrs.

In experiment II, test doses from 25 to 500 μg/mL were used. Approximately 50,000 V79 cells were seeded into 25 cm^2 culture flasks without metabolic activation. After an attachment period of approx. 48 hrs the test item was added in complete culture medium. 1 h later, 1.5 μg/mL cytochalasin B was added and the cells were incubated for 23 hrs at 37 °C. At the end of the treatment the cell culture medium was removed and the cells were prepared for microscopic analysis.

For experiment I and II, duplicate cultures were used with each dose.

2.2.3. In vivo mammalian erythrocyte micronucleus test

This study was performed at VBNCRS, India, approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) with registration number 1117/PO/RcBiBt-S/RcBiNRc-L/07/CPCSEA and AAALAC International. The Study was

Table 2
Treatment groups in the in vivo mammalian erythrocyte micronucleus test.

Group number	Group	Treatment (dose mg/kg b.w. per day)	Volume of gavage feed (mL/kg b.w. per day)
1	Vehicle control	Soya oil (-)	4.04
2	Low dose	EPAX EVOLVE 05 (600)	4.04
3	Intermediate dose	EPAX High concentrate VLC (900)	4.04
4	High dose	EPAX High concentrate VLC (1200)	4.04
5	Positive control	Cyclophosphamide monohydrate (20)	10.00
6	Positive control	Cyclophosphamide monohydrate (40)	10.00
7	Positive control	Cyclophosphamide monohydrate (60)	10.00
8	Negative control	Sterile water (-)	10.00

approved by the Institutional Animals Ethics Committee (IAEC) of the test facility under the project titled "In Vivo Mammalian Erythrocyte Micronucleus Test of VLCFA Oil (EPAX) in Sprague Dawley Rats", IAEC protocol No. VB/IAEC/03/2023/1561/Rat/SD approved on 31/03/2023. All procedures were in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of India.

The in vivo mammalian erythrocyte micronucleus test was performed in accordance with the OECD 474 guideline and performed under Good Laboratory Practice. The study was performed in Sprague Dawley rats and consisted of a main experiment with positive and negative controls and 3 dose groups of EPAX EVOLVE 05. Animals were given 2 gavage-fed doses over a 24-hr period and euthanised for bone marrow cell harvesting at 18–24 hrs after the last dosing. The positive control (Cyclophosphamide monohydrate) was given at 20 mg/kg b.w., 40 mg/kg b.w. and 60 mg/kg b.w.as a single dose. Oils containing EPAX EVOLVE 05 were prepared for gavage feeding by dilution with soya oil. Each dosing group consisted of 5 male and 5 female animals. Treatment groups are shown in Table 2.

Animals were acclimatised for at least 5 days prior to dosing and underwent a physical examination prior to randomisation. Clinical signs were observed at pre-dose, 1 hr, 2–4 hrs, and 24 hrs post dosing. Mortality and morbidity were recorded twice daily. Body weight was recorded at animal receipt, randomisation, and study days 1 and 2, prior to sacrifice.

Bone marrow cells were obtained from the femora and flushed out with foetal bovine serum solution using a syringe. Cells were centrifuged at 1500 rpm (252xg) for 10 min and resuspended in 0.1–0.2 mL and stained with May-Grunwald and Giemsa.

Cytotoxicity was determined by counting the ratio of immature and polychromatic erythrocytes vs total erythrocytes from at least 500 cells. Additionally, at least 4000 polychromatic erythrocytes were analysed for micronuclei and reported as % micro-nucleated polychromatic erythrocytes.

Statistical analysis was performed using Systat software, Mann-Whitney test was used for statistical significance testing between groups. Test items were considered clastogenic if at least one treatment group showed a statistically significant increase in micronucleated cells compared to vehicle control, the increase was dose related and results were outside of historical controls.

Doses of the test item in the study were chosen based on a NOAEL of 1200 mg/kg b.w, per day of EPAX EVOLVE 05 determined from a 90-day repeat dose toxicity study in Sprague Dawley rats.

2.2.4. Target tissue exposure

To determine tissue exposure of EPAX EVOLVE 05, plasma samples were taken from Sprague Dawley rats at 0, 2, 4, 6, and 8 hrs following a single bolus gavage dose of 1200 mg/kg b.w. Plasma samples were obtained from 6 male and 6 female rats, samples from each time point were pooled for each sex. Samples were shipped on dry ice for analysis by Epax Norway AS.

The plasma samples were prepared as follows: 1 mL of a solution containing 0.05 mg/mL C23:0 internal standard was added to a test tube and the solvent was evaporated under a stream of nitrogen. Blood plasma was then added and the weight of tissue noted. 3.5 mL of a solution containing 0.5 M sodium methoxide in methanol was added and the test tube was then heated in a boiling water bath for 1 h. After cooling, 5 mL of BCL3 was added and the test tube was heated in the boiling bath for 5 min. After heating the test tube, 0.6 mL of isooctane was added and washed with 5 mL of saturated sodium chloride in water. The iso-octane phase was transferred to micro-vials and injected directly on the GC.

Analysis was performed using a Scion 436 GC with Autosampler and Helium as carrier gas. Split/split less injection A Restek Rxi-5 ms column, 30 m, 0.25 mm ID, 0.25 μm film, was used. Temperature program: 1.5 min at 90 °C, 30 °C/min to 210 °C, 1.5 °C/min to 280 °C, 3.5 °C/min to 310 °C, isothermic at 310° for 4.6 min. C23:0 was used as an internal standard, and DHA as standard. The response factor for DHA was used to calculate a theoretical response factor for each of the VLC fatty acids. Quality control data is available. VLCFA were measured using a modified version of European Pharmacopeia method 2.4.29 "Composition of fatty acids in oils rich in omega-3-acids". C23:0 was used as an internal standard, and DHA as standard. The response factor for DHA was used to calculate a theoretical response factor for each of the VLC fatty acids. Analyses were performed in duplicate.

Table 3
Pre-experiment results from AMES test.

Substance	Dose (μL/plate)	TA98 Mutation Factor [toxicity/precipitation]		TA100 Mutation Factor [toxicity/precipitation]	
		without S9	with S9	without S9	with S9
Solvent Control (DMSO)	-	1.0	1.0	1.0	1.0
4-NOPD	10.0 μg	13.9	-	-	-
NaN_3	10.0 μg	-	-	6.4	-
2-AA	2.50 μg	-	80.9	-	17.1
Test Item	0.00316	1.1	1.1	1.0	1.1
	0.01	1.1	1.5	0.9	1.1
	0.0316	0.9	1.2	0.9	1.1
	0.1	0.8	0.9	0.9	1.0
	0.316	1.0	1.2	1.0	1.0
	1.0	0.8	1.2	1.0	1.0
	2.5	1.1	1.2	1.0	1.1
	5.0	1.0	1.0	1.0	1.1

Abbreviations: DMSO: dimethyl sulfoxide; 4-NOPD: 4-nitro-o-phenylene-diamine; 2-AA: 2-aminoanthracene

3. Results

3.1. Genotoxicity studies

3.1.1. Bacterial reverse mutation test (AMES test)

No toxicity or precipitation was noted with any dose with or without metabolic activation in the pre-experiment and corresponding doses were used in the main experiment. The results from the pre-experiment are shown in Table 3.

No precipitation was seen in any of the experimental designs. No toxic effects were seen in the pre-incubation method (experiment I). No toxic effects were seen in the pre-incubation test (experiment II) except for tester strain TA1537 at a VLCFA concentration of 0.316 and higher (without metabolic activation). No biologically relevant increases in

Table 4
Results from AMES test, Experiment I (plate incorporation) for each strain.

Experiment I – *S. typhimurium* TA98							
Treatment	Dose/ plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	46	2.3	47	4.0	1.1	1.4
DMSO	-	42	4.0	35	3.5	1.0	1.0
Test item	0.0316 µL	40	1.0	42	6.4	0.9	1.2
	0.1 µL	35	1.2	33	5.5	0.8	0.9
	0.316 µL	44	13.5	40	3.6	1.0	1.2
	1.0 µL	35	6.7	43	3.8	0.8	1.2
	2.5 µL	45	14.6	40	13.5	1.1	1.2
	5.0 µL	42	14.2	34	4.5	1.0	1.0
4-NOPD	10 µg	587	78.0	-	-	13.9	-
2-AA	2.5 µg	-	-	2789	151.6	-	80.9
Experiment I – *S. typhimurium* TA100							
Treatment	Dose/ plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	124	14.2	123	17.9	0.9	1.2
DMSO	-	137	33.0	105	18.2	1.0	1.0
Test item	0.0316 µL	129	4.7	119	11.4	0.9	1.1
	0.1 µL	121	10.7	107	11.7	0.9	1.0
	0.316 µL	136	14.4	109	6.7	1.0	1.0
	1.0 µL	132	5.6	110	11.4	1.0	1.0
	2.5 µL	131	5.3	111	10.4	1.0	1.1
	5.0 µL	133	10.1	120	14.6	1.0	1.1
NaN_3	10 µg	874	47.2	-	-	6.4	-
2-AA	2.5 µg	-	-	1800	116.2	-	17.1
Experiment I – *S. typhimurium* TA1535							
Treatment	Dose/ plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	9	2.3	10	2.1	1.4	1.9
DMSO	-	6	1.5	5	1.0	1.0	1.0
Test item	0.0316 µL	8	5.0	9	2.1	1.3	1.7
	0.1 µL	6	2.5	8	3.8	1.0	1.5
	0.316 µL	7	3.5	7	3.5	1.2	1.5
	1.0 µL	8	3.6	7	2.6	1.3	1.4
	2.5 µL	7	2.0	9	1.0	1.1	1.8
	5.0 µL	7	3.6	10	2.5	1.1	2.1
NaN_3	10 µg	1387	73.6	-	-	219.0	-
2-AA	2.5 µg	-	-	229	27.0	-	45.9
Experiment I – *S. typhimurium* TA1537							
Treatment	Dose/ plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	13	3.1	16	2.3	1.1	0.8
DMSO	-	12	2.0	21	1.0	1.0	1.0
Test item	0.0316 µL	8	3.2	14	1.5	0.7	0.7
	0.1 µL	11	1.5	12	3.1	0.9	0.6
	0.316 µL	10	2.6	15	3.0	0.8	0.7
	1.0 µL	10	6.8	20	5.3	0.9	1.0
	2.5 µL	16	1.0	10	5.6	1.3	0.5
	5.0 µL	13	2.5	14	7.5	1.1	0.7
4-NOPD	40 µg	113	6.1	-	-	9.4	-
2-AA	2.5 µg	-	-	178	12.9	-	8.5
Experiment I – *E. Coli* WP2 uvrA (pKM101)							
Treatment	Dose/ plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	328	38.6	200	26.2	1.1	1.0
DMSO	-	305	28.9	202	24.2	1.0	1.0
Test item	0.0316 µL	225	13.6	234	11.5	0.7	1.2
	0.1 µL	237	31.8	252	9.8	0.8	1.2
	0.316 µL	216	1.2	269	53–7	0.7	1.3
	1.0 µL	236	21.4	274	13.7	0.8	1.4
	2.5 µL	235	16.7	249	8.9	0.8	1.2
	5.0 µL	221	22.3	240	9.5	0.7	1.2
MMS	1 µL	1461	59.5	-	-	4.8	-
2-AA	10 µg	-	-	860	211.9	-	4.3

revertant colony numbers of any of the five tester strains were observed following treatment with VLCFA at any concentration level, neither in the presence nor absence of metabolic activation in experiment I and II. Tables 4 and 5 below summarise the data from experiment I and II, respectively.

From the mutagenicity tests performed, VLCFA did not cause gene mutations by base pair changes or frameshifts in the genome of the tester strains used.

Therefore, EPAX EVOLVE 05 is considered to be non-mutagenic in this bacterial reverse mutation assay.

3.1.2. *In vitro micronucleus test*

For the micronucleus study the VLCFA oil was diluted in DMSO. A 4-hr short term experiment (Experiment I) with and without metabolic activation, and a 24-hr long-term experiment (Experiment II) without metabolic activation were performed. In experiment I, precipitation was noted at 250 mg/mL, however no precipitation was noted in experiment II. The highest doses for experiment I were 250 and 200 mg/mL without and with metabolic activation respectively, and 500 mg/mL for experiment II. Cytotoxicity was measured and for the highest concentration should not exceed 55% ± 5%, since cytotoxicity may elicit chromosomal damage as a secondary effect. The laboratory reported that laboratory experience was used to set the cytostasis limit to 30%.

In experiment I, with and without metabolic activation, cytostasis above 30% was not noted. In experiment II without metabolic activation cytostasis above 30% was not noted up to 25 µg/mL. At 100 µg/mL a cytostasis of 33%, at 250 µg/mL a cytostasis of 46% and at 500 µg/mL a cytostasis of 49% was observed.

In experiment I with and without metabolic activation, no biologically relevant increase in the number of micronucleated cells was found. Test results for Experiment I are shown in Fig. 1 and Fig. 2 and tabulated results shown in Supplementary Tables S2 and S3.

In experiment II, biologically relevant increases in the micronuclei frequency were observed at test item concentrations of 250 and 500 µg/mL. The observed increases were statistically significant compared to the concurrent solvent control and were above historical control limits. The study therefore concluded that under the experimental conditions reported, VLCFA induced structural and/or numerical chromosomal damage in Chinese hamster V79 cells.

3.1.3. *In vivo mammalian erythrocyte micronucleus test*

In this third study for genotoxicity, no mortality or clinical signs were noted for any of the dosing groups. No significant increase in the micronucleated polychromatic erythrocytes were observed between dose groups and controls.

The VLCFA test item treated groups did not show any biologically relevant or statistically significant increase in the frequency of micronucleated polychromatic erythrocytes compared to the vehicle control group. The observed mean percentage of micronucleated polychromatic erythrocytes at the VLCFA dose levels of 600 mg/kg/day, 900 mg/kg/day and 1200 mg/kg/day was 0.27, 0.25 and 0.22 in males and 0.33, 0.25 and 0.38 in females, respectively. The observed mean percentage of

Table 5
Results the AMES test, Experiment II (pre-incubation) for each strain.

Experiment II – *S. typhimurium* TA98							
Treatment	Dose/plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	42	4.0	45	5.1	1.2	1.4
DMSO	-	35	4.9	33	6.5	1.0	1.0
Test item	0.0316 µL	23	4.0	35	8.2	0.7	1.1
	0.1 µL	27	6.4	41	4.9	0.8	1.2
	0.316 µL	25	7.8	31	8.9	0.7	0.9
	1.0 µL	31	3.8	36	4.6	0.9	1.1
	2.5 µL	23	1.7	32	5.7	0.7	1.0
	5.0 µL	38	8.5	41	2.1	1.1	1.2
4-NOPD	10 µg	542	26.6	-	-	15.6	-
2-AA	2.5 µg	–	-	1290	1044	-	39.5
Experiment II – *S. typhimurium* TA100							
Treatment	Dose/plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	139	21.9	108	18.7	1.4	1.0
DMSO	-	97	0.6	111	7.5	1.0	1.0
Test item	0.0316 µL	99	2.0	89	3.0	1.0	0.8
	0.1 µL	116	9.5	98	9.2	1.2	0.9
	0.316 µL	107	4.0	78	8.7	1.1	0.7
	1.0 µL	108	4.2	95	15.5	1.1	0.9
	2.5 µL	112	8.5	87	7.4	1.2	0.8
	5.0 µL	115	20.0	90	9.5	1.2	0.8
NaN_3	10 µg	555	104.0	-	-	5.7	-
2-AA	2.5 µg	-	-	608	101.5	-	5.5
Experiment II – *S. typhimurium* TA1535							
Treatment	Dose/plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	14	5.2	9	1.5	1.5	1.1
DMSO	-	9	3.2	8	1.5	1.0	1.0
Test item	0.0316 µL	12	5.5	8	3.8	1.3	1.0
	0.1 µL	9	4.0	9	2.6	0.9	1.1
	0.316 µL	10	2.9	10	4.5	1.1	1.2
	1.0 µL	16	2.1	10	0.0	1.7	1.2
	2.5 µL	7	1.7	9	1.5	0.8	1.0
	5.0 µL	8	2.0	10	2.1	0.9	1.2
NaN_3	10 µg	1317	72.4	-	-	141.1	-
2-AA	2.5 µg	-	-	107	74.6	-	12.8
Experiment II – *S. typhimurium* TA1537							
Treatment	Dose/plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	9	4.4	20	0.6	1.1	1.3
DMSO	-	8	1.7	15	2.1	1.0	1.0
Test item	0.0316 µL	13	5.0	15	5.0	1.7	1.0
	0.1 µL	15	2.0	18	5.3	1.9	1.2
	0.316 µL	12	2.5	13	6.0	1.5	0.8
	1.0 µL	13	3.5	12	4.9	1.6	0.8
	2.5 µL	10	1.5	10	2.9	1.3	0.7
	5.0 µL	13	2.9	15	4.6	1.6	1.0
4-NOPD	40 µg	83	8.6	-	-	10.4	-
2-AA	2.5 µg	-	-	78	39.1	-	5.1
Experiment II – *E. Coli* WP2 uvrA (pKM101)							
Treatment	Dose/plate	Revertant colonies per plate				Mutation Factor	
		Without S9		With S9			
		Mean	SD	Mean	SD	Without S9	With S9
A. dest.	-	347	16.8	381	11.6	0.8	0.9
DMSO	-	434	73.1	430	23.9	1.0	1.0
Test item	0.0316 µL	346	15.2	363	68.7	0.8	0.8
	0.1 µL	368	29.6	400	3.2	0.8	0.9
	0.316 µL	350	26.2	441	17.1	0.8	1.0
	1.0 µL	360	7.6	463	29.7	0.8	1.1
	2.5 µL	340	25.7	438	69.0	0.8	1.0
	5.0 µL	370	13.3	433	28.0	0.9	1.0
MMS	1 µL	2210	163.9	-	-	5.1	-
2-AA	10 µg	-	-	1142	119.4	-	2.7

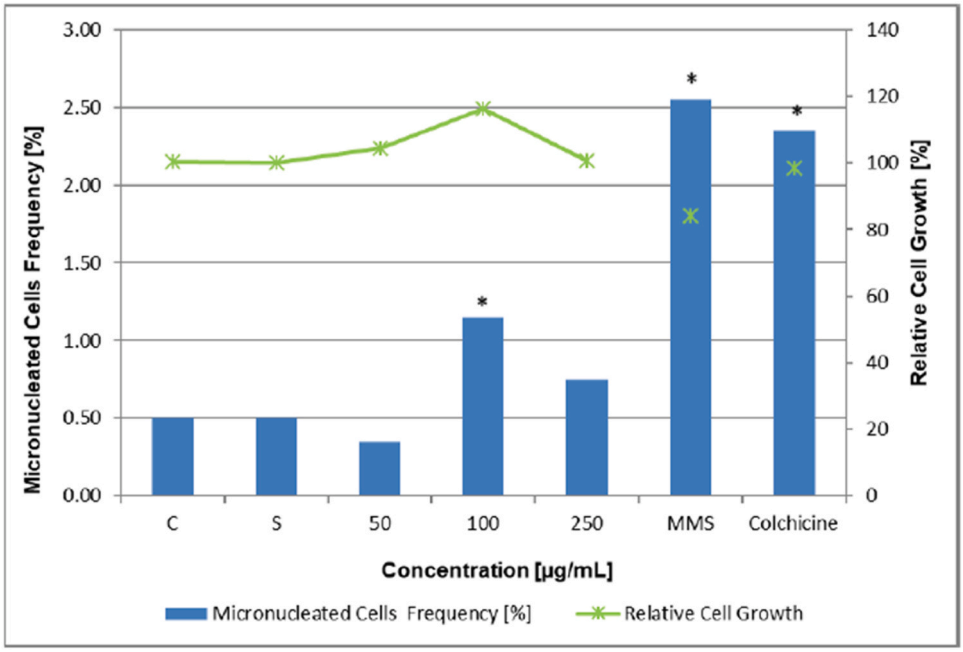

Fig. 1. In vitro micronucleus frequency and growth rate for Experiment I without metabolic activation.

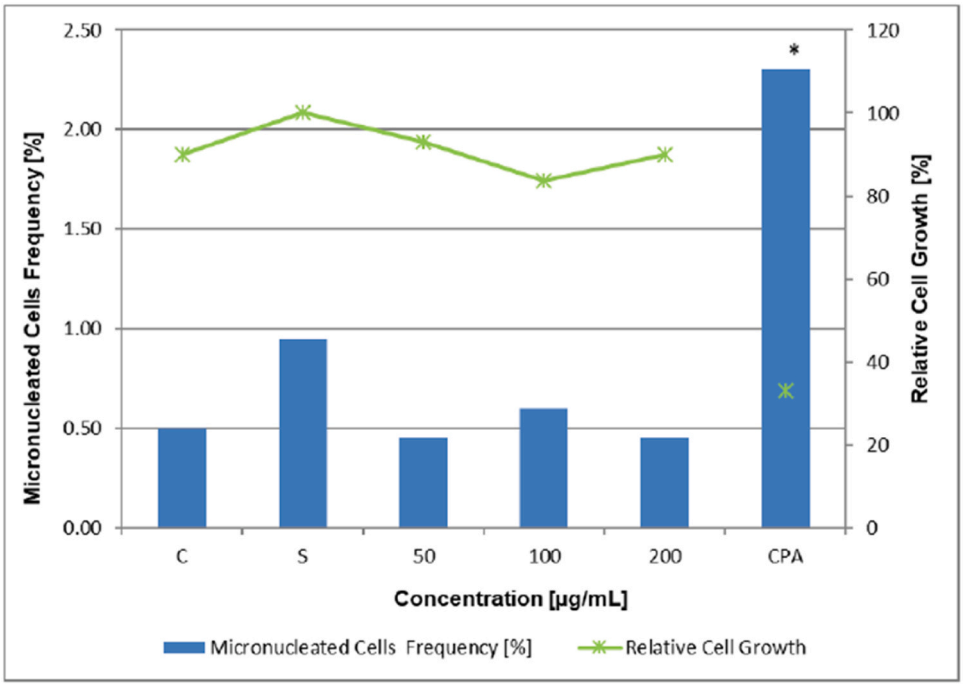

Fig. 2. In vitro micronucleus frequency and growth rate for Experiment I with metabolic activation.

micronucleated polychromatic erythrocytes in the concurrent vehicle group was 0.20 for males and 0.22 for females.

Cyclophosphamide monohydrate treatment at a dose level of 20 mg/kg/day, 40 mg/kg/day or 60 mg/kg/day induced a statistically and biologically significant increase in micronucleus frequency. The observed mean percentage of micronucleated polychromatic erythrocytes was 0.59, 0.91 and 1.07 in males and 0.63, 0.78 and 0.78 in females, respectively. The observed mean percentage of micronucleated polychromatic erythrocytes in the concurrent negative control group was 0.20 for males and 0.15 for females. The results obtained for the positive control groups and the concurrent negative control group demonstrate the sensitivity of the assay and validate the test procedures used in the study.

Table 6 presents the data for PCE/total erythrocyte % change and % change in micronuclei by dosing group. Additional data is provided in Supplemental tables S4 and S5.

Based on the results obtained, it is concluded that VLCFA does not induce micronuclei in rat bone marrow cells under the tested conditions. Therefore, EPAX EVOLVE 05 is considered non-mutagenic in the micronucleus test under tested conditions.(Figs. 3, 4, 5, 6).

Table 6
In vivo micronuclei results.

Male			
Group	Dose	% change PCE/ Total	%MN (SD)
1: Vehicle control	-	NA	0.20 (0.07)
2: Low dose VLC	600 mg/kg	2.89	0.27 (0.04)
3: Intermediate dose VLC	900 mg/kg	2.16	0.25 (0.06)
4: High dose VLC	1200 mg/ kg	-1.51	0.22 (0.09)
5: Positive control low dose	20 mg/kg	14.20	0.59 * (0.17)
6: Positive control intermediate dose	40 mg/kg	16.94	0.91 * (0.10)
7 Positive control high dose	60 mg/kg	18.04	1.07 * (0.18)
8 Negative control	Water	NA	0.20 (0.04)
Female			
Group	Dose	PCE/Total	%MN
1: Vehicle control	-	NA	0.22 (0.12)
2: Low dose VLC	600 mg/kg	0.35	0.33 (0.07)
3: Intermediate dose VLC	900 mg/kg	-0.13	0.25 (0.02)
4: High dose VLC	1200 mg/ kg	0.15	0.38 (0.17)
5: Positive control low dose	20 mg/kg	14.34	0.63 * (0.15)
6: Positive control intermediate dose	40 mg/kg	15.29	0.78 * (0.12)
7 Positive control high dose	60 mg/kg	19.75	0.78 * (0.13)
8 Negative control	Water	NA	0.15 (0.04)

%change of PCE/Total for test item is calculated as a comparison to the vehicle control, % change of PCE/total for positive control samples as a comparison to the negative control. Significance was only calculated for %MN. * =$p < 0.05$

3.2. VLC fatty acids in blood plasma

The following VLC fatty acids were detected and analysed in blood plasma: MUFA: C24:1 and C26:1; PUFA: C24:4, C24:5, C24:6, C26:4, C26:5, C26:6, C26:7, C28:4, C28:5, C28:8, C30:6 and C30:8. A graphical representation of total MUFA (panel A), total PUFA (panel B), and total VCLFAs (panel C) are shown below.

The data show that there is a very low level of MUFA and PUFA prior to dosing with VLC fatty acids. Uptake into blood reached a maximum at 2 h for males and 4 h for females. Calculated absorption parameters are shown in the table below(Table 7).

4. Discussion

Fatty acids described in this study are naturally occurring in fish, present mostly as triglyceride molecules (in-house data). In commercial long chain fatty acid concentrates from fish oil, and in the proposed EPAX EVOLVE 05, fatty acids are present as re-constituted triglycerides [7]. These have the same molecular form as natural oils, but there are a greater number of desired fatty acids on each glycerol molecule [7]. Therefore, the absorption, distribution, metabolism, and toxicity characteristics of EPAX EVOLVE 05 are likely to reflect that of long chain fatty acid concentrates.

EPA and DHA have been widely studied for their benefits and potential side effects and the FDA has set an upper dietary limit for adult consumption of 3 g EPA+DHA daily [23], whilst EFSA has a 5 g limit [22]. Oils containing EPA and DHA also contain a host of additional lipids that are co-purified. In Epax´ analysis of its own oils, an anchovy concentrate was shown to contain around 20 mg/g of VLCFA, this being 10-times less than that of EPAX EVOLVE 05 (not published). This concentration difference prevents extrapolation of safety data from conventional omega-3 oils but does provide an indication that low levels of VLCFA intake is safe. As previously stated, whole fish also contain VLCFA, and portions taken during meals provide similar levels of VLCFA as 2 g of EPAX EVOLVE 05. Thus, there is background evidence that fish and fish oil products containing VLCFA are safe and without known toxicity.

No mutagenicity was seen with the bacterial reverse mutation test (AMES test), with or without metabolic activation. In a subsequent in

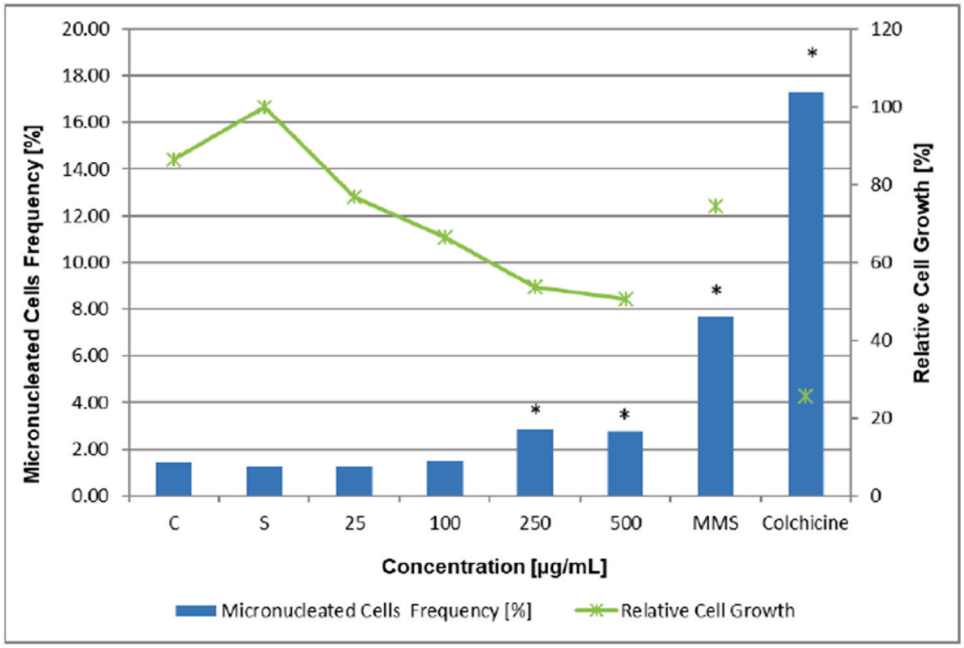

Fig. 3. In vitro micronucleus frequency and growth rate for Experiment II without metabolic activation.

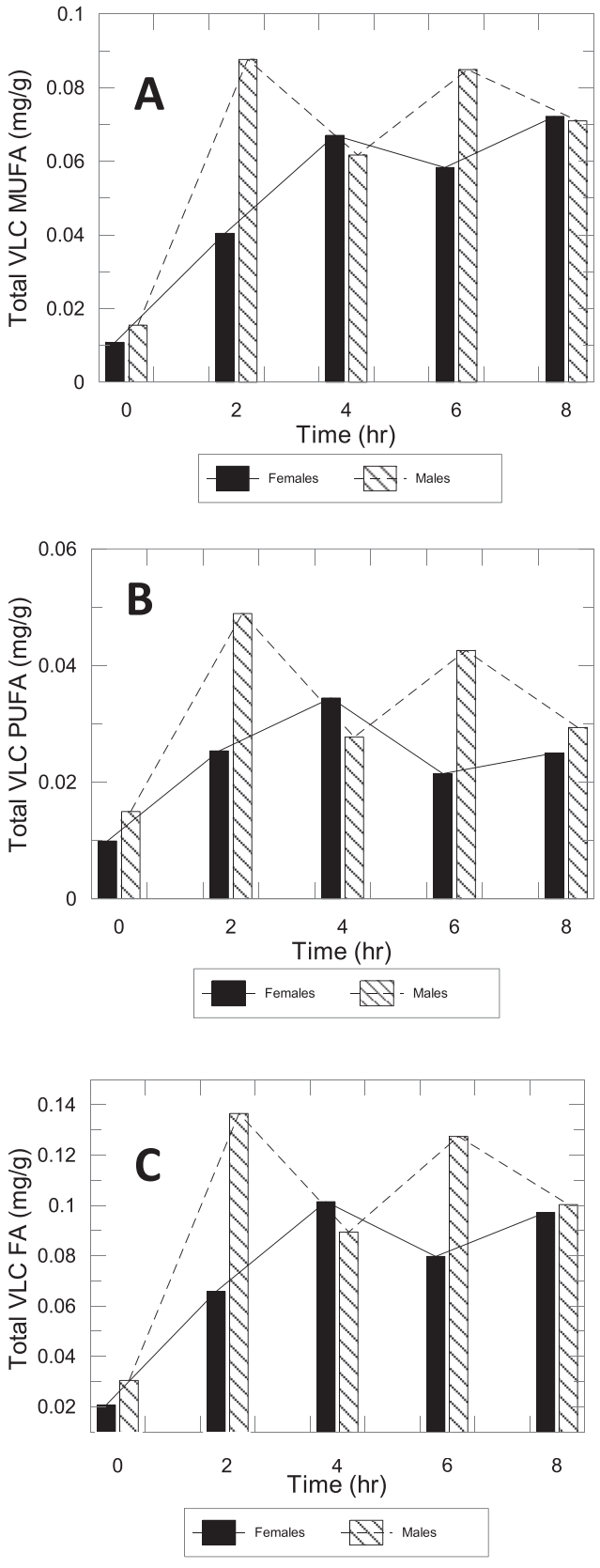

Fig. 4. Blood plasma levels of total VLC MUFA (panel A), total VLC PUFA (panel B), or total VLCFA (panel C).

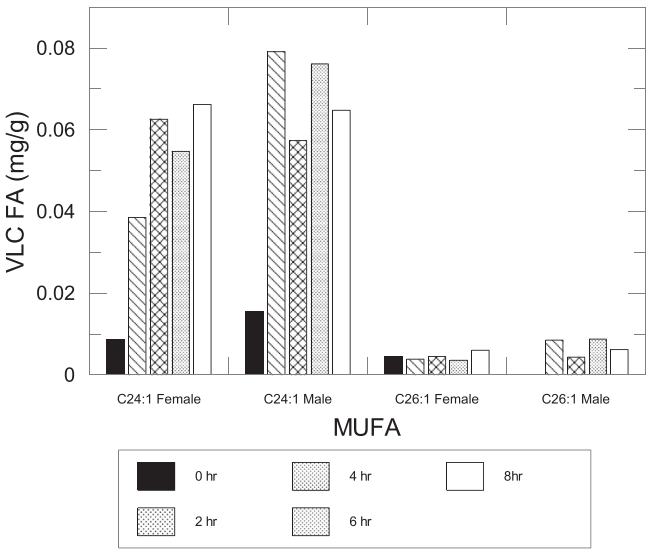

Fig. 5. Blood plasma concentration (mg/g) of MUFA C24:1 and C26:1 fatty acids following oral gavage administration.

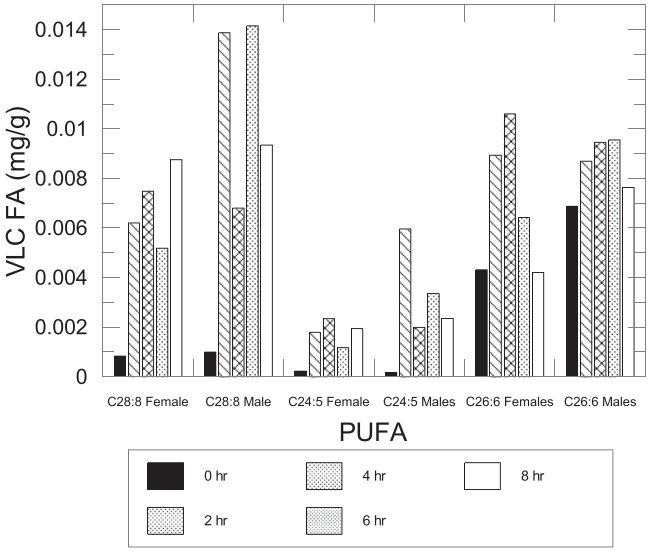

Fig. 6. Blood plasma concentration (mg/g) of PUFA C24:5, C26:6, and C28:8 fatty acids following oral gavage administration.

vitro micronucleus study performed at 4 hrs and 24-hrs duration, a positive signal was seen with the 24-hr study without metabolic activation. The study criteria for mutagenicity were met although it is noteworthy that the cytotoxicity of the two highest doses was 46% and 49% bordering the 50% cut-off value defined in the protocol. In addition, abnormally high cytostasis was noted.

Thus, to investigate further the genotoxic potential of VLCFAs, an in vivo micronucleus study was performed in Sprague Dawley rats. The dosage regimen reflected recent findings from a 90-day repeat-dose study with EPAX EVOLVE 05 oil using a maximum dose of 1200 mg VLCFA / kg b.w. The maximum dose for the in vivo genotoxicity was set to the same amount, and a 2-day exposure chosen to capture repeat-dose effects. No mutagenic activity was seen with any concentration in the in vivo analysis of Sprague Dawley rats.

VLCFA were detected in blood plasma following a single bolus administration, supporting that bone marrow erythrocytes are exposed to the test item. The peak plasma level as measured in this study was between 2 and 4 hrs. In a similar study, serum samples from WT mice fed

Table 7
Target tissue absorption characteristics.

PK parameter	MUFA male	MUFA female	PUFA male	PUFA female	VLC FA male	VLC FA female
AUC $_{0\rightarrow8}$	0.5547 mg-hr/g	0.4144 mg-hr/g	0.2826 mg-hr/g	0.1973 mg-hr/g	0.8372 mg-hr/g	0.6117 mg-hr/g
Cmax	0.08761 mg/g	0.07219 mg/g	0.04891 mg/g	0.03442 mg/g	0.1365 mg/g	0.1014 mg/g
Tmax	2 hr	8 hr	2 hr	4 hr	2 hr	4 hr

a bolus of 6 mg/kg C36 VLCFA (C36:6 n-3) showed a peak uptake of C36 at 2 h with the majority of uptake being between 2 and 8 h after bolus feeding [11].

Our results are also in keeping with findings that fish oil supplementation did not result in genotoxic effects and can actually provide protection from genotoxic effects in in vivo models of rats, dogs and humans [15,18,20].

Overall, the results obtained from a series of genotoxicity studies indicate that VLCFAs do not have genotoxic potential at the concentrations tested (up to 1200 mg / kg b.w.). The presence of VLCFAs was shown in plasma samples providing evidence of tissue exposure to the test item.

CRediT authorship contribution statement

The work described in the manuscript submitted with the above titled was fully funded by Epax Norway AS. No funds, grants, or other support were received during the preparation of this manuscript. The authors Derek Tobin, Iren Stoknes and Harald Svensen are employees of Epax Norway AS. Michael Dornish is an independent toxicology expert used as a consultant by Epax Norway AS. Derek Tobin is the project manager for the performance of the toxicology studies. Derek Tobin and Michael Dornish as the main authors with contributions for chemistry and analysis and oil production from Harald Svensen. Iren Stoknes has performed proof reading and study design advice. No humans were used in these studies. The in vivo rat study was performed in India. All animal studies were performed after approval from relevant ethics committees. The study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) with registration number 1117/PO/RcBiBt-S/RcBiNRc-L/07/CPCSEA and AAALAC International. The Study was approved by the Institutional Animals Ethics Committee (IAEC) of the test facility under the project titled “In Vivo Mammalian Erythrocyte Micronucleus Test of VLC FA Oil (EPAX) in Sprague Dawley Rats”, IAEC protocol No. VB/IAEC/03/2023/1561/Rat/SD approved on 31/03/2023. All procedures were in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of India.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Derek Tobin reports financial support was provided by Epax Norway AS. Derek Tobin reports a relationship with Epax Norway AS that includes: employment. Iren Stoknes and Harald Svensen are employees of Epax Norway AS. Michael Dornish is a consultant for Epax Norway AS.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2023.09.009.

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