Lipids
DOI 10.1007/s11745-016-4189-y

ORIGINAL ARTICLE

Wax Ester Rich Oil From The Marine Crustacean, *Calanus finmarchicus*, is a Bioavailable Source of EPA and DHA for Human Consumption

Chad M. Cook[1] · Terje S. Larsen[2] · Linda D. Derrig[1] · Kathleen M. Kelly[1] · Kurt S. Tande[3]

Received: 28 April 2016 / Accepted: 18 August 2016

Abstract Oil from the marine copepod, *Calanus finmarchicus*, which contains >86 % of fatty acids present as wax esters, is a novel source of n-3 fatty acids for human consumption. In a randomized, two-period crossover study, 18 healthy adults consumed 8 capsules providing 4 g of Calanus® Oil supplying a total of 260 mg EPA and 156 mg DHA primarily as wax esters, or 1 capsule of Lovaza® providing 465 mg EPA and 375 mg DHA as ethyl esters, each with an EPA- and DHA-free breakfast. Plasma EPA and DHA were measured over a 72 h period (t = 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 h). The positive incremental area under the curve over the 72 h test period ($iAUC_{0–72\ h}$) for both EPA and DHA was significantly different from zero ($p < 0.0001$) in both test conditions, with similar findings for the $iAUC_{0–24\ h}$ and $iAUC_{0–48\ h}$, indicating the fatty acids were absorbed. There was no difference in the plasma $iAUC_{0–72\ h}$ for EPA + DHA, or DHA individually, in response to Calanus Oil *vs* the ethyl ester condition; however, the $iAUC_{0–48\ h}$ and $iAUC_{0–72\ h}$ for plasma EPA in response to Calanus Oil were both significantly increased relative to the ethyl ester condition ($iAUC_{0–48\ h}$: 381 ± 31 *vs* 259 ± 39 μg*h/mL, $p = 0.026$; $iAUC_{0–72\ h}$: 514 ± 47 *vs* 313 ± 49 μg*h/mL, $p = 0.009$). These data demonstrate a novel wax ester rich marine oil is a suitable alternative source of EPA and DHA for human consumption.

✉ Chad M. Cook
chad.cook@mxns.com

[1] Biofortis Clinical Research, 211 East Lake St, Addison, IL 60101, USA

[2] Cardiovascular Research Group, Department of Medical Biology, Faculty of Health Sciences, UiT the Arctic University of Norway, Tromsø, Norway

[3] Calanus AS, Tromsø, Norway

Keywords n-3 Fatty acids · Fat absorption · Lipid absorption · Waxes

Abbreviations

DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
HDL-C	High-density lipoprotein cholesterol
iAUC	Incremental area under the curve
LDL-C	Low-density lipoprotein cholesterol
n-3 PUFA	Omega-3 polyunsaturated fatty acids
C_{max}	Maximal concentration
non-HDL-C	Non-high-density lipoprotein cholesterol
SDA	Stearidonic acid
TAG	Triacylglycerol(s)
TC	Total cholesterol
T_{max}	Time to maximal concentration

Introduction

The omega-3 polyunsaturated fatty acids (n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in the human diet are derived primarily from a combination of oily fish (e.g., salmon, tuna, mackerel, or herring), fortified foods, and dietary supplements. The form of EPA and DHA in many commercially available products varies, and some studies indicate the absorption, and possibly subsequent bioavailability, of EPA and DHA may differ depending on the source of the oil and corresponding chemical composition of the fatty acids. For instance, in many fish oils, EPA and DHA are primarily found in the triacylglycerol (TAG) form or as ethyl esters. In contrast, a majority of the fatty acids in krill oil are bound to phospholipids [1]. In general, short-term (e.g., ≤72 h) studies suggest the TAG form is better absorbed (typically measured

Published online: 07 September 2016

as concentration of EPA and/or DHA in the total plasma lipid pool), relative to ethyl esters, although results have been mixed, especially in comparisons of TAG and phospholipid forms of EPA and DHA [2–6].

A novel source of EPA and DHA for human consumption is oil from the marine copepod *Calanus finmarchicus*, which is the most abundant crustacean in the North Atlantic Ocean with annual production of several hundred million tonnes [7]. The oil extracted from *C. finmarchicus* is ruby colored and slightly viscous, with >86 % of the fatty acids present as wax esters bound predominantly to aliphatic long-chain monounsaturated alcohols [mostly 20:1(n-9) and 22:1(n-11) alcohols], with minor amounts of free fatty acids, free fatty alcohols, and glycerides. The oil from *C. finmarchicus* also contains phytosterols and astaxanthin, which are either not present, or found in very small amounts, in most other marine oils. To the best of our knowledge, the absorption of EPA and DHA as predominantly wax esters from *C. finmarchicus* remains to be determined in humans. Wax esters have generally been considered to be poorly digested in mammals, although little direct evidence is actually available in humans [8, 9]. Therefore, the primary objective of this study was to determine if EPA and DHA as wax esters from oil extracted from *C. finmarchicus* and consumed as an encapsulated dietary supplement (Calanus® Oil; Calanus AS, Tromsø, Norway) were absorbed in healthy men and women.

Table 1 Fatty acid profile of Calanus oil and ethyl ester oil

Fatty acid (common name)	Calanus oil (g/100 g)	Ethyl ester oil (g/100 g)
14:0 (Myristic acid)	10.7	
15:0 (Pentadecylic acid)	0.5	
16:0 (Palmitic acid)	5.0	
16:1 (Palmitoleic acid)	1.5	
16:3	0.1	
17:0 (Margaric acid)	0.2	
18:0 (Stearic acid)	0.4	
18:1 n-9 (Oleic acid)	0.3	
18:2 n-6 (Linoleic acid)	0.5	
18:3 n-3 (Alpha-linolenic acid)	0.7	
18:4 n-3 (Stearidonic acid)	7.3	
18:4 n-9	1.4	
20:1 n-9	1.5	
20:4 n-6 (Arachidonic acid)	0.2	
20:5 n-3 (Eicosapentaenoic acid)	6.5	46.5
22:1 n-11	3.0	
22:5 n-3 (Docosapentaenoic acid)	0.3	
22:6 n-3 (Docosahexaenoic acid)	3.9	37.5
24:1 n-9	0.4	

All subjects consumed 8 capsules containing Calanus oil (Calanus AS, Tromso, Norway) supplying a total of 4 g of oil providing 260 mg EPA and 156 mg DHA primarily as wax esters, or 1 capsule supplying 1 g of oil providing 465 mg EPA and 375 mg DHA as ethyl esters (Lovaza®, GlaxoSmithKline, Research Triangle Park, NC) in a randomized, crossover design

Subjects and Methods

Study Design

This randomized, two-period crossover study included one screening/baseline visit followed by two 72 h test periods, each separated by a minimum 7 day washout. Subjects that met all entry criteria at the screening/baseline visit were randomly assigned to receive 8 capsules containing Calanus oil (Calanus AS, Tromso, Norway) supplying a total of 4 g of oil providing 260 mg EPA and 156 mg DHA primarily as wax esters (fatty acid profile on a g/100 g basis presented in Table 1), or 1 capsule supplying 1 g of oil providing 465 mg EPA and 375 mg DHA as ethyl esters (Lovaza®, GlaxoSmithKline, Research Triangle Park, NC). Hence, the amounts of EPA and DHA provided in the ethyl ester oil were ~ 1.8 and 2.4 fold higher, respectively, than the amount of EPA and DHA provided in Calanus Oil. After a minimum 7 day washout, all subjects crossed over to receive the opposite study product at the beginning of the second 72 h test period.

This study was conducted at Biofortis Clinical Research (d.b.a. Biofortis, Inc., Addison, IL) according to Good Clinical Practice (GCP) Guidelines (US 21 Code of Federal Regulations) and the Declaration of Helsinki (2000). This study was registered at clinicaltrials.gov (NCT02865044) and the protocol was approved by an accredited Institutional Review Board (IntegReview IRB, Austin, TX). All participants provided signed informed consent and authorization for disclosure of protected health information before any study specific procedures were carried out.

On the first morning of each 72 h test period, subjects arrived at the clinic between approximately 0600 and 0800 hours following an overnight fast (12 ± 1 h; water only) to provide a blood sample to determine baseline plasma EPA and DHA status ($t = -0.5$ h timepoint). All subjects then consumed their assigned study product with a standardized EPA- and DHA-free breakfast, containing ~ 23 g total fat (not including study products) based on a 2500 kcal/day diet, over a 15 min timeframe. Following study product and breakfast consumption ($t = 0$ h), blood samples were obtained (via an indwelling venous catheter or venipuncture if the catheter failed) at $t = 1, 2, 4, 6, 8, 10, 12, 24, 48$, and 72 h ± 5 min for measurements of plasma EPA and DHA fatty acid concentrations over time. Thus, blood samples from $t = 1$–12 h represented postprandial

measurements on the first day of each test period after product consumption, while blood samples at $t = 24$, 48, and 72 h were collected in the morning of each test period following overnight fasts (12 ± 1 h; water only).

Standardized, low-fat, EPA- and DHA-free lunch and dinner meals were provided on the first day of each 72 h test period immediately following $t = 4$ h and the $t = 8$ h blood draws; both meals were consumed in entirety within 15 min. A standardized, low-fat, EPA- and DHA-free snack was administered at $t = 11$ h. Food intake was based on each subject's estimated energy needs for weight maintenance using 30 kcal/kg body weight per day [10]. The menus and time of food consumption for breakfast, lunch, dinner, and the snack were the same for each test period.

Subjects were instructed to maintain their habitual dietary practices, physical activity patterns, and body weight, but to limit alcohol intake to one drink per day (1 drink = 12 oz beer, 5 oz wine, or 1½ oz distilled spirits) and to avoid fish/seafood and other EPA- or DHA-containing foods and supplements during each test period. Compliance with these instructions was monitored through the use of daily diet records completed by each subject during each test period. Subjects were also asked to avoid vigorous exercise for 24 h prior to all test visits, which was confirmed verbally the morning of each clinic visit.

Subjects

Men and non-pregnant, non-lactating women, 18–59 years of age (inclusive), each with a body mass index (BMI) 18.50–29.99 kg/m^2 and a fasting TAG concentration <200 mg/dL, who were in good general health on the basis of medical history and routine laboratory tests were eligible for the study. In addition, subjects were required to be willing to refrain from consumption of all fish and seafood (including shellfish), fatty acid-containing supplements, and/or EPA-, DHA-containing foods and supplements for 14 days prior to randomization. The use of any medications, dietary supplements, or fortified foods with lipid-altering effects was excluded for at least 4 weeks before study entry, as was a recent (within 3 months of screening) change in body weight >4.5 kg. Additionally, individuals were excluded from participation if they used non-study related omega-3 fatty acid drug(s) or dietary supplement(s) containing ≥1.0 g/d of EPA, DHA, or a combination of EPA and DHA within 4 months of screening. Individuals with a known allergy or sensitivity to omega-3 fatty acids, fish, other seafood, or any ingredient in the study products or meals were also excluded.

Additional exclusion criteria included resting systolic blood pressure ≥160 mmHg and/or a diastolic blood pressure ≥100 mm Hg, and history or presence of clinically important endocrine (including type 1 or 2 diabetes mellitus), cardiovascular (including, but not limited to history of myocardial infarction, peripheral arterial disease, stroke), pulmonary (including uncontrolled asthma), hepatic, renal, hematologic, immunologic, dermatologic, neurologic, psychiatric or biliary disorders.

Laboratory Methods

Analyses of serum lipoprotein lipids, plasma chemistry, and whole-blood hematology samples for screening purposes were performed by Elmhurst Memorial Hospital (EMH) Reference Laboratory (Elmhurst, IL) according to their standard validated procedures, including the Standardization Program of the Centers for Disease Control and Prevention and the National Heart, Lung and Blood Institute for lipid measurements [11]. Lipoprotein lipid assessments (mg/dL) included total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), non-HDL-C (calculated as TC minus HDL-C), TAG, and the TC/HDL-C ratio. The LDL-C concentration in mg/dL was calculated according to the Friedewald equation as: LDL-C = TC − HDL-C − TAG/5 [12].

Plasma fatty acid concentration was analyzed by OmegaQuant (Sioux Falls, SD) via gas chromatography (GC) with flame ionization detection. Blood samples were obtained via EDTA-treated (lavender top) vacutainers [Becton–Dickinson (BD), New Jersey, USA]. Following refrigerated centrifugation at 1500 ×g for 15 min, 1.0 mL aliquots of plasma were transferred to polypropylene cryovials using a Pasteur pipette and stored frozen at −80 °C prior to batch analysis. Plasma for analysis was transferred to a screw-cap glass vial which contained 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine as in internal standard (di-C17:0 PL) (Avanti Polar Lipids, USA) and the methylation reagent (methanol containing 14 % boron trifluoride, toluene, methanol; 35:30:35 v/v/v; Sigma-Aldrich, St. Louis, MO) was added. The vial was briefly vortexed and heated in a hot bath at 100 °C for 45 min. After cooling, hexane (EMD Chemicals, USA) and HPLC grade water were added, the tubes were recapped, vortexed and centrifuged to separate layers. An aliquot of the hexane layer was transferred to a GC vial. GC was carried out using a GC-2010 Gas Chromatograph (Shimadzu Corporation, Columbia, MD) equipped with a SP-2560, 100-m fused silica capillary column (0.25 mm internal diameter, 0.2 μm film thickness; Supelco, Bellefonte, PA).

Fatty acids were identified by comparison with a standard mixture of fatty acids (GLC OQ-A, NuCheck Prep, Elysian, MN) which was also used to determine individual fatty acid calibration curves. The di-C17:0 PL was used to calculate recovery efficiency of the assay and applied to all fatty acids. Fatty acid composition was expressed as a

percent of total identified fatty acids and concentrations as μg/mL of plasma.

Statistical Analyses

Statistical analyses were performed using SAS (SAS Institute, Cary, NC, version 9.3). Sample size calculations were completed for the comparison of the incremental area under the curve (iAUC) to a null value of zero using a single-sample *t* test. Assuming a standard deviation of 17.2 μg*h/mL, based on prior studies completed in our laboratory, it was determined a sample of 15 subjects would provide 80 % statistical power to detect a mean iAUC value of ≥13.5 μg*h/mL ($\alpha = 0.05$, two-sided). Eighteen subjects were randomized to allow for possible attrition.

Analyses were completed for both an efficacy evaluable sample population, which included all subjects who were randomized and provided at least one post-randomization outcome data point during each test period, and a per protocol sample population defined as a subset of the efficacy evaluable subjects that did not have any major protocol violations. All study samples were defined prior to locking the database. All tests of significance were performed at $\alpha = 0.05$, two-sided.

Baseline comparability of treatment sequence groups for screening variables were assessed by analysis of variance (ANOVA) or Chi-square tests, as appropriate. The primary outcome variable was the iAUC for plasma EPA + DHA from pre-product consumption ($t = -0.5$ h) to 72 h ($iAUC_{0–72\ h}$) during each test condition. Secondary outcomes included kinetic parameters [maximal concentration (C_{max}) and time to C_{max} (T_{max})] and the iAUC over 24, 48, and 72 h for each individual plasma fatty acid and the combination of EPA + DHA (except as indicated in the primary outcome variable).

Within each test condition, the *t* test was used to determine if the $iAUC_{0–72\ h}$ for plasma EPA + DHA was significantly different from zero. Repeated measures analysis of covariance (ANCOVA) was used to assess differences between test conditions for the primary and secondary outcome variables. Initial repeated measures ANOVA models contained terms for intervention, sequence and period, with subject as a random effect and sex as a covariate. Models were reduced using a backward selection method until only significant terms or intervention remained in the model (sex remained in the final model regardless of its coefficient *p*-value).

Assumptions of normality of residuals and homogeneity of variances were investigated for each outcome variable using the Shapiro–Wilk test [13] and Levene's test [14], respectively. If either the normality assumption was rejected at the 1 % level or the homogeneity of variances assumption at the 5 % level, then an analysis of rank-transformed data was performed.

Safety assessments included an evaluation of intervention-emergent adverse events compared between the two test conditions. Additionally, frequencies of scores of 3 or 4 (somewhat more than usual and much more than usual) were tabulated for individual gastrointestinal symptoms (gas/bloating, nausea, vomiting, abdominal cramping, abdominal distention/bloating, borborygmus/stomach rumbling, burping, and reflux) from a gastrointestinal tolerability questionnaire. McNemar's test was used to assess differences in frequencies across test conditions before and after each test period.

Results

Subjects

A total of 18 subjects were randomized and all subjects completed the study, representing the efficacy evaluable sample population (Fig. 1). A per protocol analysis ($n = 16$) was also performed excluding data from two subjects that had important protocol deviations. One subject was documented as a non-smoker during the medical history review at screening visit, but during the study self-reported smoking at least 3 cigarettes a month. This subject claimed to not use cigarettes or nicotine products while involved in the study, but this was determined to be an important deviation and warranted exclusion of this subject from the per protocol analysis. A second subject inadvertently ate salmon and avocado sushi (9 pieces) for lunch during one of the test periods, which was also deemed an important protocol deviation and warranted exclusion of this subject from the per protocol analysis.

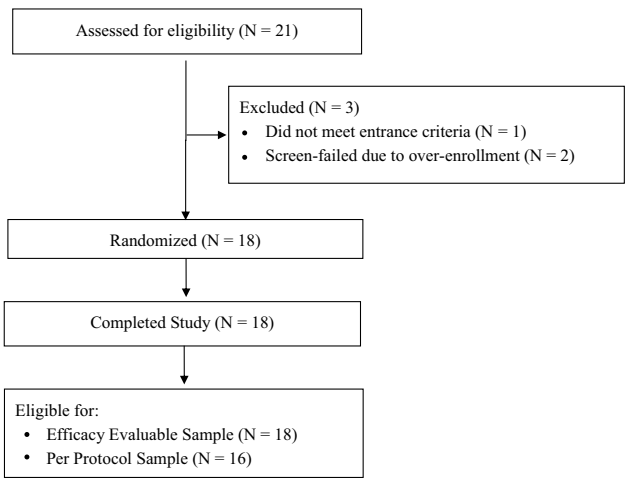

Fig. 1 Disposition of subjects

Table 2 Baseline characteristics of subjects

Characteristic	Efficacy evaluable sample ($N = 18$) n (%)
Sex	
Male	9 (50.0)
Female	9 (50.0)
Race	
White	13 (72.2)
Black/African American	1 (5.6)
Asian/Pacific Islander	3 (16.7)
Other	1 (5.6)
	Mean ± SEM
Age (years)	38.3 ± 2.5
Weight (kg)	73.1 ± 3.1
Body mass index (kg/m^2)	25.1 ± 0.6
Fasting plasma triacylglycerols (mg/dL)	97.2 ± 10.1

Results for both sequences were pooled

SEM standard error of the mean

The data presented represent the 18 subjects included in the efficacy evaluable analysis as the results were not materially different from the per protocol analysis. Baseline demographics of the efficacy evaluable sample population are presented in Table 2. Data are reported as mean ± standard errors of the mean (SEM) or median and interquartile range (IQR) unless otherwise indicated.

Plasma EPA and DHA Response to Calanus Oil and EE Oil Consumption

The 72 h time course of plasma EPA + DHA, EPA, and DHA in response to a single dose of ethyl ester oil and Calanus oil is shown in Fig. 2a–c and the corresponding kinetic parameters are presented in Table 3. The $iAUC_{0–72\ h}$ was significantly different from zero ($p < 0.0001$) in both test conditions, with similar findings for the $iAUC_{0–24\ h}$ and $iAUC_{0–48\ h}$, indicating the fatty acids were absorbed from both oils. There was no difference in the plasma EPA + DHA $iAUC_{0–72\ h}$ ($p = 0.219$) or DHA $iAUC_{0–72\ h}$ ($p = 0.499$) in response to Calanus oil relative to the ethyl ester oil. Interestingly, whereas plasma EPA reached a plateau after about 6 h during the ethyl ester oil condition (Fig. 2b), plasma EPA continued to rise in response to Calanus oil and remained elevated for the rest of the observation period (compared to the ethyl ester oil test condition). Consistent with this finding, the plasma EPA $iAUC_{0–48\ and\ 0–72\ h}$ were each significantly increased ($p \leq 0.026$ and $p \leq 0.009$, respectively) in response to

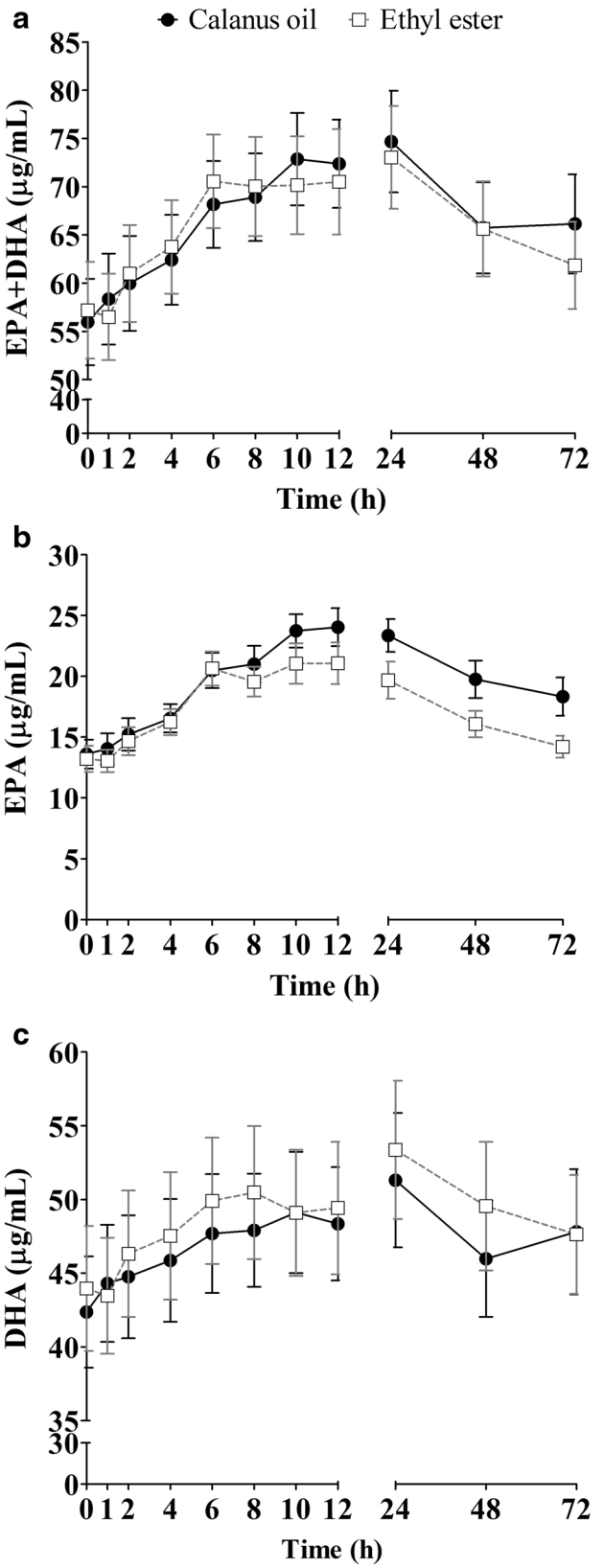

Fig. 2 The 72-h time course of plasma EPA + DHA (**a**), EPA (**b**), and DHA (**c**) in response to a single dose of Calanus oil or ethyl ester formulation. Data presented as means ± SEM. *DHA* docosahexaenoic, *EPA* eicosapentaenoic acid

Table 3 Kinetic parameters for plasma EPA + DHA, EPA, and DHA in response to a single serving of Calanus oil and ethyl ester oil

Parameter[a]	Ethyl ester oil	Calanus oil	*P* value[b]
EPA + DHA			
$iAUC_{0–72\ h}$ (μg*h/mL)	764 ± 93	931 ± 92	0.219
$iAUC_{0–48\ h}$ (μg*h/mL)	585 ± 63	681 ± 61	0.291
$iAUC_{0–24\ h}$ (μg*h/mL)	291 ± 32	335 ± 30	0.320
C_{max} (μg/mL)	77 ± 5	80 ± 5	0.705
T_{max} (h)	16.4 ± 2.7	20.3 ± 3.9	0.392
EPA			
$iAUC_{0–72\ h}$ (μg*h/mL)	313 ± 49	514 ± 47	0.009
$iAUC_{0–48\ h}$ (μg*h/mL)	259 ± 39	381 ± 31	0.026
$iAUC_{0–24\ h}$ (μg*h/mL)	146 ± 20	190 ± 14	0.088
C_{max} (μg/mL)	23 ± 2	26 ± 2	0.205
T_{max} (h)	17.3 ± 4.1	16.9 ± 3.5	0.716
DHA			
$iAUC_{0–72\ h}$ (μg*h/mL)	460 ± 66	438 ± 75	0.499
$iAUC_{0–48\ h}$ (μg*h/mL)	327 ± 41	308 ± 46	0.767
$iAUC_{0–24\ h}$ (μg*h/mL)	146 ± 18	150 ± 21	0.884
C_{max} (μg/mL)	55 ± 5	55 ± 5	0.951
T_{max} (h)	27.4 ± 5.5	27.4 ± 5.4	0.936

C_{max} maximum concentration, *DHA* docosahexaenoic, *EPA* eicosapentaenoic acid, *h* hour, *iAUC* incremental area under the curve, *SEM* standard error of the mean, T_{max} time to reach C_{max}

[a] Results for both treatment sequences were pooled for all parameters and represent data from the efficacy evaluable analysis. Data presented as mean ± SEM

[b] *P* value for Calanus oil *vs*. ethyl ester oil

Calanus oil relative to ethyl ester oil, with a trend for a significant difference in the plasma EPA $iAUC_{0–24}$ ($p = 0.088$) between conditions. It should be noted that the increased iAUC values for EPA in response to Calanus oil occurred despite the fact that the amount of EPA given in the form of Calanus oil (260 mg) was considerably less than that given as ethyl ester oil (465 mg). There were no statistically significant differences between test conditions in T_{max} or C_{max}.

Safety Analyses

Frequencies of scores of 3 or 4 (somewhat more than usual and much more than usual) for gastrointestinal symptoms (gas/flatulence, nausea, vomiting, abdominal cramping, abdominal distention/bloating, borborygmus/stomach rumbling, burping, and reflux/heartburn) were not significantly different at any time (beginning, middle, end) during each test period (data not shown; $p > 0.05$ for all).

There was a statistically significant change in body weight during the first test period (baseline = 73.3 ± 3.0 *vs* 73.0 ± 3.0 kg, $p = 0.0475$), with a trend for a significant change in weight during the second test period (73.6 ± 3.1 *vs* 73.3 ± 3.0 kg, $p = 0.0845$). These mean changes in weight over each 72 h test period did not appear to be clinically meaningful as the values were within normal biological day-to-day fluctuations commonly observed in similar clinical trials, as the average percent change from baseline weight was <3 %. No adverse events were considered serious or severe, and none were considered possibly, probably, or definitely related to study product consumption by the study physicians.

Discussion

The principle finding from this randomized, crossover study demonstrates that EPA and DHA as predominantly wax esters in Calanus oil are digested and absorbed in generally healthy men and women, as evidenced by statistically significant increases in the concentration of each of these n-3 PUFA in the total plasma lipid pool over a 72 h period. The results from this study are novel, as EPA and DHA in most alternative commercially available sources are found predominantly as TAG, ethyl ester, or phospholipid forms.

These findings are of particular interest, as wax esters have generally been considered to be poorly digested in mammals, although little direct evidence is actually available in humans [8, 9]. Wax esters *per se* are a normal part of the diet as a lipid component of certain foods, including unrefined whole grain cereals, seeds, and nuts [8]. Wax esters are also consumed in considerable amounts by certain populations that regularly eat fish roe [15] or certain fish species, such as orange roughy [16]. However, it is unlikely that wax esters are consumed in considerable quantities in regions of the world eating a diet consisting of highly refined and processed foods [8]. While the mechanism of TAG digestion and absorption has been well studied, less consideration has been given to the same processes underlying metabolism of dietary waxes in humans. Lipases and carboxyl esterases that hydrolyze TAG have demonstrated enzymatic activity towards wax esters [8], but a lipase specific for wax ester digestion has not been identified in mammals [17]. *In vitro* data suggest the rate of wax ester hydrolysis by purified porcine pancreatic lipase is 10–50 times slower compared to TAG hydrolysis due in part to product inhibition and hydrophobicity of wax esters [18, 19]. This is also consistent with digestion of ethyl esters, as the ethyl ester bond is resistant to the effects of pancreatic lipase *in vitro*, with the enzyme efficiency for ethyl ester bonds being only about 2 % of that for TAG and diacylglycerol bonds. The rate of wax ester hydrolysis is one factor determining the rate of absorption of the corresponding fatty acids, which influences bioavailability. Indeed, based on the kinetic data observed in the

current study, it appears EPA and DHA provided as wax esters reaches a maximal concentration at approximately 20 h post-consumption, which was numerically higher compared to the ethyl ester oil condition albeit not statistically significantly different. Whether the rate of hydrolysis of wax esters is lower than that of ethyl esters remains to be determined and may be of importance only if the level of wax esters is consumed in substantially higher amounts than provided in the present study (4 g).

To the best of our knowledge, this is the first human study to-date examining the absorption of EPA and DHA from a wax ester rich marine oil. Thus, only *in vitro* experiments and a few animal studies are available for direct comparison. The complete digestion of synthesized and naturally occurring (jojoba oil) wax esters rich in EPA and DHA has been demonstrated *in vitro* following 24 h incubation with a combination of human pancreatic lipase, porcine pancreatic colipase, and deoxycholic acid [20]. Within 4 to 6 h, approximately 80 % of the wax esters were hydrolyzed, with approximately 50 % of the total fatty acids released. After 24 h, the wax esters in each test condition were completely hydrolyzed and 100 % of the fatty acids, chiefly EPA and DHA, were released. Goretta and colleagues [20] have also demonstrated that EPA and DHA wax esters, consumed for 4 weeks, are incorporated into plasma phospholipids of rats to a similar extent as TAG or ethyl ester formulations, providing evidence that fatty acids as wax esters can be absorbed in mammals. Recent work in apolipoprotein E-deficient mice has shown that, despite consuming diets containing similar amounts of EPA and DHA, blood levels of both EPA and DHA were significantly higher in mice fed a diet supplemented with Calanus oil compared to those fed an EPA + DHA ethyl ester enriched diet [21]. Furthermore, Calanus oil has been observed to have beneficial effects on obesity-related abnormalities in rodent models of diet-induced obesity at EPA and DHA fatty acid concentrations considerably lower than the concentrations used in similar earlier studies using other sources of EPA and DHA [22, 23]; indicating EPA and DHA as wax esters have to be digested, absorbed, and assimilated to exert biological effects. Taken together, based on the available *in vitro* data, animal data, and the findings of the present study demonstrating that circulating concentrations of EPA and DHA remained elevated up to 72 h after a single serving of 4 g of Calanus oil, the hydrolyzed products of wax ester digestion are most likely slowly absorbed *in vivo*.

Many studies have examined the absorption of EPA and DHA from different sources and in different chemical forms (reviewed in [2–4]). The first human studies by Lawson and Hughes [24, 25] indicated better absorption of both EPA and DHA (intakes of 1.00 and 0.67 g, respectively) in the TAG form compared to the ethyl ester form. However, these investigators also demonstrated that the difference in absorption between the two forms was less pronounced after a high-fat meal compared to a low-fat meal. Thus, in our current study, the total fat content at breakfast (co-ingested with study products) was approximately 25 g based on a 2500 kcal/day diet to account for this. Subsequent short-term studies have also indicated that EPA and DHA provided as ethyl esters are less bioavailable compared to TAG forms [26–28], but not all studies support this [29, 30] and differences in study designs make comparisons challenging. However, a very recent study added to this body of literature has demonstrated no significant differences in mean fasting n-3 fatty acid plasma concentrations in response to 1.3 g/d of EPA + DHA from fish oil ethyl esters, fish oil TAG, or phospholipid rich krill oil after 4 weeks of supplementation [5]. Furthermore, the authors noted that during the first 48 h, there was nearly identical absorption kinetics of EPA + DHA between the three study products.

A criticism of past human studies comparing absorption of EPA and/or DHA from different lipid sources has been the use of different amounts of fatty acids between products. In the present study, the amounts of EPA and DHA were considerably lower with intake of Calanus oil compared to ethyl esters. This is also a potential limitation of the present study, but adjustment for the lower EPA and DHA intakes associated with Calanus oil would suggest that the wax ester form is better absorbed. One of the interesting findings from this study was that plasma EPA concentrations over the 72 h test period were significantly higher in response to Calanus oil in comparison to a well-defined ethyl ester formulation (Lovaza), even though the amount of EPA provided in Calanus oil was approximately half (56 %) of the amount of EPA provided as ethyl esters (260 *vs* 465 mg). The rise in plasma EPA approximately 8 h after intake of Calanus oil suggests delayed uptake, probably due to slow release and absorption in the distal part of the intestine [31]. Another possibility is metabolic conversion of wax ester-derived stearidonic acid (SDA) to EPA [32, 33]. That 4 g of Calanus oil provides approximately 300 mg of SDA could at least partially explain the observed plasma EPA response to Calanus oil; however, this was not directly determined in this study.

In conclusion, Calanus oil appears to be a suitable alternative source of EPA plus DHA to help meet the daily intake recommendations for long chain n-3 PUFA. Additional research examining the absorption of EPA and DHA as wax esters in different fasting and post-prandial lipid pools, as well as dose response studies, would be of future value. More work is also warranted to directly compare absorption of EPA and DHA as wax esters in Calanus oil to other forms of EPA and DHA, including TAG and phospholipid forms.

Acknowledgments The authors would like to thank Margie Huebner of ClinData Services (Fort Collins, CO) and Hua Kern of Biofortis

Clinical Research for assistance in planning and performing statistical analyses.

Compliance with Ethical Standards

Conflict of Interest This was a sponsored study funded by Calanus AS (Tromsø, Norway). At the time the study was conducted, C.M. Cook and L.D. Derrig were employees of Biofortis Clinical Research, and K. Tande was an employee of Calanus AS. T. Larsen has received research support from Calanus AS.

References

1. Gigliotti JC, Davenport MP, Beamer SK, Tou JC, Jaczynski J (2011) Extraction and characterisation of lipids from Antarctic krill (*Euphausia superba*). Food Chem 125:1028–1036
2. Ghasemifard S, Turchini GM, Sinclair AJ (2014) Omega-3 long chain fatty acid "bioavailability": a review of evidence and methodological considerations. Prog Lipid Res 56:92–108
3. Salem N Jr, Kuratko CN (2014) A reexamination of krill oil bioavailability studies. Lipids Health Dis 13:137
4. Schuchardt JP, Hahn A (2013) Bioavailability of long-chain omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 89:1–8
5. Yurko-Mauro K, Kralovec J, Bailey-Hall E, Smeberg V, Stark JG, Salem N Jr (2015) Similar eicosapentaenoic acid and docosahexaenoic acid plasma levels achieved with fish oil or krill oil in a randomized double-blind four-week bioavailability study. Lipids Health Dis 14:99
6. Cook CM, Hallaraker H, Saebo PC, Innis SM, Kelley KM, Sanoshy KD, Berger A, and Maki KC (2016) Bioavailability of long chain omega-3 polyunsaturated fatty acids from phospholipid-rich herring roe oil in men and women with mildly elevated triacylglycerols. Prostaglandins Leukot Essent Fatty Acids 111:17–24
7. Melle W, Ellertsen B, Skjoldal HR (2004) Zooplankton: the link to higher trophic levels. In: Skjoldal HR (ed) The Norwegian sea ecosystem. Tapir Academic Press, Trondheim
8. Hargrove JL, Greenspan P, Hartle DK (2004) Nutritional significance and metabolism of very long chain fatty alcohols and acids from dietary waxes. Exp Biol Med (Maywood) 229:215–226
9. Place AR (1992) Comparative aspects of lipid digestion and absorption: physiological correlates of wax ester digestion. Am J Physiol 263:R464–R471
10. Human energy requirements: report of a joint FAO/WHO/UNU Expert Consultation (2005) Food Nutr Bull 26:166
11. Myers GL, Cooper GR, Winn CL, Smith SJ (1989) The centers for disease control-national heart, lung and blood institute lipid standardization program. an approach to accurate and precise lipid measurements. Clin Lab Med 9:105–135
12. Friedewald WT, Levy RI, Fredrickson DS (1972) Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 18:499–502
13. Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611
14. Levene H (1960) Robust tests for the equality of variance. In: Olkin I (ed) Contributions to probability and statistics: essays in honor of Harold Hotelling, 1st edn. Stanford University Press, Palo Alto, pp 278–292
15. Bledsoe GE, Bledsoe CD, Rasco B (2003) Caviars and fish roe products. Crit Rev Food Sci Nutr 43:317–356
16. De Koning AJ (2005) Phospholipids of marine origin : the orange roughy (*Hoplostethus atlanticus*). S Afr J Sci 101:414–416
17. Ling KH, Nichols PD, But PP (2009) Fish-induced keriorrhea. Adv Food Nutr Res 57:1–52
18. Savary P (1971) The action of pure pig pancreatic lipase upon esters of long-chain fatty acids and short-chain primary alcohols. Biochim Biophys Acta 248:149–155
19. Lee RF, Patton JS (1989) Alcohol and waxes. In: Ackman RG (ed) Marine biogenic lipids, fats, and oils. CRC Press, Boca Raton
20. Gorreta F, Bernasconi R, Galliani G, Salmona M, Tacconi MT, Bianchi R (2002) Wax esters of n-3 polyunsaturated fatty acids: a new stable formulation as a potential food supplement. 1—digestion and absorption in rats. Lebensm-Wiss und Technol 35:458–465
21. Eilertsen KE, Maehre HK, Jensen IJ, Devold H, Olsen JO, Lie RK, Brox J, Berg V, Elvevoll EO, Osterud B (2012) A wax ester and astaxanthin-rich extract from the marine copepod *Calanus finmarchicus* attenuates atherogenesis in female apolipoprotein E-deficient mice. J Nutr 142:508–512
22. Hoper AC, Salma W, Khalid AM, Hafstad AD, Sollie SJ, Raa J, Larsen TS, Aasum E (2013) Oil from the marine zooplankton *Calanus finmarchicus* improves the cardiometabolic phenotype of diet-induced obese mice. Br J Nutr 110:2186–2193
23. Hoper AC, Salma W, Sollie SJ, Hafstad AD, Lund J, Khalid AM, Raa J, Aasum E, Larsen TS (2014) Wax esters from the marine copepod *Calanus finmarchicus* reduce diet-induced obesity and obesity-related metabolic disorders in mice. J Nutr 144:164–169
24. Lawson LD, Hughes BG (1988) Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters. Biochem Biophys Res Commun 152:328–335
25. Lawson LD, Hughes BG (1988) Absorption of eicosapentaenoic acid and docosahexaenoic acid from fish oil triacylglycerols or fish oil ethyl esters co-ingested with a high-fat meal. Biochem Biophys Res Commun 156:960–963
26. Dyerberg J, Madsen P, Moller JM, Aardestrup I, Schmidt EB (2010) Bioavailability of marine n-3 fatty acid formulations. Prostaglandins Leukot Essent Fatty Acids 83:137–141
27. el Boustani S, Colette C, Monnier L, Descomps B, Crastes de Paulet A, Mendy F (1987) Enteral absorption in man of eicosapentaenoic acid in different chemical forms. Lipids 22:711–714
28. Schuchardt JP, Schneider I, Meyer H, Neubronner J, von Schacky C, Hahn A (2011) Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations–a comparative bioavailability study of fish oil vs. krill oil. Lipids Health Dis 10:145
29. Nordoy A, Barstad L, Connor WE, Hatcher L (1991) Absorption of the n-3 eicosapentaenoic and docosahexaenoic acids as ethyl esters and triglycerides by humans. Am J Clin Nutr 53:1185–1190
30. Krokan HE, Bjerve KS, Mork E (1993) The enteral bioavailability of eicosapentaenoic acid and docosahexaenoic acid is as good from ethyl esters as from glyceryl esters in spite of lower hydrolytic rates by pancreatic lipase in vitro. Biochim Biophys Acta 1168:59–67
31. Verschuren PM, Nugteren DH (1989) Evaluation of jojoba oil as a low-energy fat. 2. Intestinal transit time, stomach emptying and digestibility in short-term feeding studies in rats. Food Chem Toxicol 27:45–48
32. Maki KC, Rains TM (2012) Stearidonic acid raises red blood cell membrane eicosapentaenoic acid. J Nutr 142:626S–629S
33. Whelan J, Gouffon J, Zhao Y (2012) Effects of dietary stearidonic acid on biomarkers of lipid metabolism. J Nutr 142:630S–634S