Lipids (2016) 51:39–48
DOI 10.1007/s11745-015-4086-9

ORIGINAL ARTICLE

Dietary ALA from Spinach Enhances Liver n-3 Fatty Acid Content to Greater Extent than Linseed Oil in Mice Fed Equivalent Amounts of ALA

Miho Kuroe[1] · Hiroyuki Kamogawa[1] · Masashi Hosokawa[1] · Kazuo Miyashita[1]

Received: 2 August 2015 / Accepted: 13 October 2015 / Published online: 5 November 2015

Abstract Although several works have reported absorption rate differences of n-3 polyunsaturated fatty acids (PUFA) bound to different lipid forms, such as ethyl ester, triacylglycerol (TAG), and phospholipids, no studies have investigated the effect of n-3 PUFA from glycolipids (GL). The present study compared the fatty acid contents of tissue and serum lipids from normal C57BL/6J mice fed two types of α-linolenic acid (ALA)-rich lipids, spinach lipid (SPL), and linseed oil (LO). ALA was primarily present as the GL form in SPL, while it existed as TAG in LO. Supplementation of both lipids increased ALA and its n-3 metabolites, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid, and decreased n-6 PUFA, linoleic acid and arachidonic acid, in the livers, small intestines, and sera of the treated mice compared with those of the control group. When the comparison between the SPL and LO diets containing the same amount of ALA was conducted, the EPA and DPA levels in the liver lipids from mice fed the SPL diet were significantly higher than those fed the LO diet. Additionally, the total contents of n-3 PUFA of lipids from the livers, small intestines, and sera of the SPL group were higher than those of the LO group.

Keywords Glycolipids · α-Linolenic acid absorption · Eicosapentaenoic acid · Docosapentaenoic acid · Arachidonic acid

✉ Kazuo Miyashita
kmiya@fish.hokudai.ac.jp

[1] Laboratory of Bio-functional Material Chemistry, Division of Marine Bioscience, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido 041-8611, Japan

Abbreviations

ALA	α-Linolenic acid
ARA	Arachidonic acid
DGDG	Digalactosyl diacylglycerol
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EE	Ethyl esters
EPA	Eicosapentaenoic acid
FFA	Free fatty acid
GC	Gas chromatography
GL	Galactolipids
LA	Linoleic acid
LO	Linseed oil
MGDG	Monogalactosyl diacylglycerol
PC	Phosphatidyl choline
PL	Phospholipids
SPL	Spinach lipids
SQDG	Sulfoquinovosyl diacylglycerol
TAG	Triacylglycerol
TL	Total lipids
TLC	Thin layer chromatography

Introduction

Plant leaves generally contain up to 7 wt% total lipids (TL) per dry weight [1]. We have analyzed TL levels of 13 types of commercial green leafy vegetables harvested in Hokkaido, Japan, and found that the variation in the TL ranged from 6.1 to 13.0 wt% per dry weight [2]. This TL level was relatively higher than those of fruits, flowers, and stem vegetables (2–4 wt% per dry weight) (unpublished data). The higher TL level of green leafy vegetables is due to the large amounts of thylakoid membrane lipids in the tissues. The major constituents of leaf lipids are monogalactosyl

diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) with moderate amounts of sulfoquinovosyl diacylglycerol (SQDG) and phospholipids (PL), while only small amounts of neutral lipids are present [3]. The high levels of MGDG and DGDG in the leaf lipids originate from the unusually high composition of both galactolipids (GL) in the thylakoid membranes of chloroplasts [4]. Usually, MGDG and DGDG from plant leaf lipids have very high amounts of α-linolenic acid (18:3n-3, ALA) [1]. The ALA content of acylated fatty acids has been reported to be approximately 95 % in MGDG from leaf chloroplasts of cucurbits [5], alfalfa [6], wheat [7], and green holly [8], while that in DGDG has been reported in the range from 79 to 88 %. Thus, green leafy vegetable lipids are potential dietary sources of ALA.

ALA is an essential fatty acid that must be consumed through the diet. There have been many epidemiological and clinical studies on the cardiovascular-protective effects of ALA [9]. ALA is a precursor of eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids. Both n-3 EPA and DHA have sometimes been regarded as active forms of ALA in biological systems. EPA and DHA have been shown to cause significant biochemical and physiological changes in the body that often result in a positive influence on human nutrition and health. EPA and DHA consumption have benefits of reducing the risk of cardiovascular disease, probably due to regulation of membrane structure, lipid metabolism, blood clotting, blood pressure, and inflammation [10–14]. Thus, the bioconversion of ALA to EPA and DHA is important for understanding the biological importance of ALA.

Humans have been generally considered to have a poor ability to form DHA from ALA. Tracer studies have shown that the proportion of ALA conversion to DHA in infants is very low, less than 1 % [15]. Another study has demonstrated that in adult men, the conversion of ALA to EPA is limited (approximately 8 %), and conversion to DHA is extremely low (<0.1 %) [16]. However, studies in normal healthy adults consuming Western diets showed that supplemental ALA raised EPA and DPA statuses in the blood and breast milk. Addition of ALA to the diets of formula-fed infants has been shown to raise DHA levels [17]. Another study showed that there was no difference in brain DHA accretion between rats fed ALA and DHA [18]. This was due to decreased DHA metabolism and an increased rate of DHA synthesis in rats fed ALA. The conversion of ALA to DHA by the liver and other specific DHA-requiring tissues, such as the brain, provides ample DHA when sufficient ALA has been consumed [19]. Thus, the need for ALA is extremely apparent because ALA is by far the predominant form of n-3 PUFA consumed in the typical Western and vegetarian diets [20].

ALA is present in notable amounts in plant sources, including green leafy vegetables and commonly consumed oils, such as rapeseed and soybean oils. Additionally, ALA-rich oils, such as flaxseed oil, are commercially available. These ALA-containing products are a major source of n-3 PUFAs, especially in Western and vegetarian diets. ALA in seed oils exists as triacylglycerol (TAG), while in green leafy vegetables most ALA is bound to GLs, such as MGDG and DGDG. MGDG and DGDG digestion is known to be based on lipase hydrolysis of pancreatic juice, similar to TAG digestion [21, 22]; however, the ALA absorption as these GL may be different from that of TAG because several studies have reported different absorption rates of EPA and DHA in different ester forms, such as TAG, PL, and ethyl esters (EE) [23–28]. Among these, EPA and DHA of PL have been reported to show the highest bioavailability, followed by those of TAG and EE [25–27]. Higher absorption rates of EPA and DHA derived from PL have been reported in a human study using krill oil as a dietary lipid [24, 28].

Although the intake of each PUFA from the different dietary form such as EE, TAG, and PL have been investigated, no study has been performed on those lipids bound to GL. Thus, in the present study, the intake of ALA from GL was compared with that from TAG using spinach leaf as a source of ALA-rich GL.

Materials and Methods

Standards and Chemicals

Standard MGDG, DGDG, and SQDG were purchased from Lipid Products (Redhill, UK), while standard phosphatidyl choline (PC) was from Avanti Polar Lipids Inc. (Alabaster, AL, USA). All of the other chemicals and solvents used in this study were of analytical grade.

Separation and Analysis of Spinach Leaf Lipids

Dried spinach leaf powder (GABAN Co. Ltd., Tokyo, Japan) was obtained from a local food market. The spinach powder (2 kg) was extracted with six volumes (v/w) of methanol. The methanol extracts were dissolved into a separatory funnel using a chloroform/methanol/water (10:5:3, v/v/v) solution. After being shaken, the funnel was allowed to stand overnight, and the lower layer was concentrated under vacuum using a rotary evaporator. The last traces of organic solvent and water were removed in a desiccator under high vacuum to obtain spinach lipids (SPL). The lipid class profile of SPL was analyzed by thin layer chromatography (TLC). The lipid fraction was dissolved in a chloroform/methanol/water (65:25:4,

v/v/v) solution and spotted onto 0.25 mm silica gel plates (Merck, Darmstadt, Germany). The plates were developed with a chloroform–methanol–water (65:25:4, v/v/v) solution, and the spots were visualized by spraying the plates with orcinol sulfuric acid or Dittmer reagent, followed by charring. The lipid samples were also analyzed by silica gel TLC using *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as the developing solvent. The spot was detected using 50 % aqueous sulfuric acid charring. The chromatogram was taken with a charge coupled device camera, and then the digital image of the plate was acquired and transferred to the computer. The image was properly cropped and saved in bitmap format using Vistascan software on a Windows-controlled system (Hewlett–Packard, Tokyo, Japan). The ratio of each lipid fraction in the sample was expressed as the bitmap percentage of the total bitmap intensities.

The fatty acid compositions of SPL and linseed oil (LO) used as dietary lipids were determined by gas chromatography (GC) after converting fatty acyl groups in the lipids to their corresponding methyl esters by transesterification using sodium methoxide (CH_3ONa) as the catalyst [29]. Briefly, 1 mL of *n*-hexane and 0.2 mL of 2 N NaOH in methanol were added to an aliquot of sample lipids, vortexed and incubated at 50 °C for 30 s. Next, 0.2 mL of 2 N HCl in methanol solution was added to the solution and mixed. The upper hexane layer was recovered and subjected to GC analysis. GC analysis was performed on a Shimadzu GC-14B instrument (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column [Omegawax 320 (30 m × 0.32 mm i.d.); Supelco, Bellefonte, PA, USA]. The injection port and flame ionization detector were set at 250 and 260 °C, respectively; the column temperature was maintained at 200 °C. The carrier gas was helium at a flow rate of 50 kPa. The fatty acid contents in lipid samples were expressed as the weight percentages of total fatty acids. For the reference, fresh leafy vegetables obtained in the local market in Hakodate, Japan, were extracted with chloroform/methanol/water (10:5:3, v/v/v) after freezed-drying, treatment and the fatty acid composition of the lipids was analyzed as described above.

Animals and Diets

The aim of this study was to compare the SPL and LO as dietary ALA source under normal conditions. Thus, normal and healthy C57BL/6J mice were used the rodent model. A total of 27 normal and healthy C57BL/6J mice (4 weeks old, male) were purchased from Charles River Laboratories (Japan, Inc., Yokohama, Kanagawa, Japan). All mice were housed in stainless cages (seven mice per a cage, four cages in total) and acclimatized for 2 weeks on a normal rodent diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan). Mice had free access to food and tap water. Room temperature and humidity were controlled at 23 ± 1 °C and 50 ± 10 % with a 12 h light/12 h dark cycle. In most animal experiments using C57BL/6 J mice, more than six mice were used. The mice were, therefore, randomly divided into four groups of seven mice in one cage and then fed experimental diets for 4 weeks. The body weight, diet, and water intake of each mouse was recorded every day. The compositions of the diets are shown in Table 1. All dietary components except for lipids were obtained from CLEA (Japan, Inc. Tokyo, Japan). Lard was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Corn oil and LO were obtained from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). For the preparation of SPL containing diet, SPL and lard were dissolved in chloroform. After mixing well, the solvent was completely removed. The mixed lipid was heated at 50 °C, and then the liquid was mixing with other dietary components. The two experimental diets, SPL (3 %) and LO, were designed to contain equal amounts of ALA, equating to approximately 0.7 g/g of the diet (Table 2). However, the resulting manufactured diets had slightly different of total fatty acid intake because of the difference in the non-acyl component levels of SPL and LO. By GC analysis using 17:0 as an internal standard, fatty acid content of the SPL (3 %) diet was 5.2 g/7 g lipids, while that of the LO diet

Table 1 Composition (g/kg) of experimental diets

Diet ingredient	Group			
	Control	LO	SPL (1 %)	SPL (3 %)
Corn starch	397.486	397.486	397.486	397.486
Dextrinized corn starch	132	132	132	132
Casein	200	200	200	200
Sucrose	100	100	100	100
Cellulose (KC flock)	50	50	50	50
AIN93G mineral mix	35	35	35	35
AIN93G vitamin mix	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
t-Butylhydroquinoe	0.014	0.014	0.014	0.014
Lard	50	55	60	40
Corn oil	20	0	0	0
Linseed oil (LO)	0	15	0	0
SPL	0	0	10	30

Spinach leaf lipids (SPL) was obtained from dried spinach leaf powder by solvent extraction described in the "Materials and Methods" section. It is mainly composed of galactolipids (GL)

Table 2 Fatty acid content of total fatty acids and total diet

	Group			
	Control	LO	SPL (1 %)	SPL (3 %)
Wt% per total fatty acids				
14:0	1.14	1.28	1.46	1.22
16:0	21.44	21.57	24.67	23.10
18:0	10.25	11.45	12.75	10.59
18:1n-9	38.85	37.56	39.44	33.03
18:1n-7	2.25	2.48	2.69	2.39
18:2n-6	2.02	9.40	7.92	8.74
18:3n-3	0.63	10.27	3.99	12.71
Grams per 100 g diet				
14:0	0.08	0.09	0.09	0.06
16:0	1.50	1.46	1.55	1.21
18:0	0.72	0.78	0.80	0.09
18:1n-9	2.72	2.55	2.48	0.56
18:1n-7	0.16	0.17	0.17	1.73
18:2n-6	1.41	0.64	0.50	0.13
18:3n-3	0.04	0.70	0.25	0.67

was 6.8 g/7 g lipids. All procedures for the use and care of animals for this research were approved by the ethical committee of Experimental Animal Care at Hokkaido University.

Fatty Acid Compositions of Dietary Lipids and ALA Levels of Each Diet

Dietary lipids were extracted from each diet with a chloroform/methanol (2:1, v/v) solution after being prepared as described previously by Folch et al. [30]. The chloroform/methanol solution contained a known amount of 17:0 as an internal standard. Fatty acid compositions of the lipids were analyzed by GC after converting fatty acyl groups in the lipids to their methyl esters, as described above. The ALA levels of the diets were calculated by comparing the peak ratios of ALA to that of the internal standard (17:0) and the lipid content.

Sample Collections

Blood samples were taken from the caudal vena cava of the mice. Mice were euthanized, and each tissue was immediately excised and weighed. The livers were immediately stored in RNA later™ (Sigma Chemical Co., St. Louis, MO, USA) for quantitative real-time PCR analysis. Blood serum analyses were conducted by the Analytical Center of Hakodate Medical Association (Hakodate, Japan). The analyses included measuring the following parameters: neutral lipids, free fatty acids, phospholipids, total cholesterol, HDL cholesterol, LDL cholesterol, and free fatty acids.

Tissue and Blood Lipid Analysis

Livers, small intestines, and brains were extracted with a chloroform/methanol (2:1, v/v) solution containing a known amount of internal standard (17:0), as described previously by Folch et al. [30]. The tissue samples from each mouse were analyzed separately. The major fatty acid contents of each tissue were analyzed by GC after converting fatty acyl groups in the lipid to their methyl esters, as described above. The contents were reported as milligrams per gram tissue. However, only small amounts of serum samples remained after the lipid parameter analyses. Therefore, all serum samples were combined in each group, and then the lipids were extracted with a chloroform/methanol/water (1:2:0.8, v/v/v) solution, as described previously by Bligh and Dyer [31]. Fatty acid compositions (wt% of total fatty acids) were analyzed by GC after converting fatty acyl groups in the lipid to their methyl esters, as described above.

Quantitative Real-Time PCR

Total RNA was extracted from the livers of mice using RNeasy Lipid Tissue Mini Kits (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real-time PCR analyses of individual cDNA were performed with ABI Prism 7500 (Applied Biosystems Japan Ltd., Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan). The mRNA analyses were performed on genes associated with the bioconversion of ALA to DHA, which included Δ^6-desaturase (Fads2) (from ALA to 18:4n-3 and 24:5n-3 to 24:6n-3), elongase-5 (Elov5) (from 18:4n-3 to 20:4n-3), Δ^5-desaturase (Fads1) (from 20:4n-3 to EPA), elongase-2 (Elov2) (from EPA to DPA and DPA to 24:5n-3), and peroxisomal acyl CoA oxidase (Acox1) (from 24:6n-3 to DHA). The gene-specific primers Mm00507605_m1 (Fads1 mRNA), Mm00517221_m1 (Fads2 mRNA), Mm00517086_m1 (Elov2 mRNA), Mm00506717_m1 (Elov5 mRNA), Mm01246834_m1 (Acox1 mRNA), and Mm99999915_g1 (GAPDH mRNA; internal control), respectively.

Statistical Analysis

Data are presented as the mean ± SEM (n = 6 or 7). Analysis of variance (ANOVA) was used to test for significant differences between different groups. Statistical

comparisons were performed using Scheffe's *F* test. Differences with $P < 0.05$ were considered significant.

Results

Fatty Acid Contents of Dietary Lipids

The major fatty acids of SPL were ALA (53.2 %), 16:0 (13.9 %), and linoleic acid (18:2n-6, LA) (12.3 %). Analysis of other leafy vegetable lipids showed the highest content of ALA in the fatty acids: Komatsuna (*Brassica rapa* var. *perviridis*), 52.5 %; Mizuna (*Brassica rapa* var. *laciniifolia*), 50.8 %, Perilla (*Perilla frutescens* var. *acuta*), 52.3 %; Sweet basil (*Ocimum basilicum*), 56.8 %; Mistuba (*Cryptotaenia japonica*), 35.0 %; Parsley (*Petroselium crispum*), 30.5 %; Garland chrysanthemum (*Chrysanthemum coronarium*), 68.3 %; Garlic chives (*Allium tuberosum*), 48.6 %; Welsh onion (*Allium fistulosum*), 47.2 %. These leafy vegetables contained 6.1–13.0 wt% lipids per dry weight and most of lipids composed of GL rich in ALA.

LO also contained a high level of ALA (45.3 %), followed by 18:1n-9 (25.3 %), LA (16.3 %), and 16:0 (5.9 %). However, the lipid class compositions of LO and SPL were different. TLC analysis showed that LO was mainly composed of TAG; however, only a small amount of TAG was detected in SPL. When each lipid composition of SPL was roughly analyzed based on the spot intensities of TLC, the main lipid class of SPL was found to be DGDG (22.0 %), followed by MGDG (17.3 %), SQDG (13.5 %), chlorophylls (11.6 %), PC (3.5 %), and lutein (1.3 %). Chlorophylls and lutein are non-acyl lipids. MGDG, DGDG, SQDG, and PC are diacyl glycerols with non-acyl components bound to the remaining position of glycerol, resulting in the relatively lower percentage of fatty acids in SPL than in LO. Therefore, for the comparison of the dietary SPL with that from LO, LO (15 g/1 kg diet) was added to the diet so that the ALA level of the diet was almost the same as that in the diet containing SPL (3 %) (Table 2).

Fatty Acid Levels of Livers (Table 3), Small Intestines (Table 4), Brains (Table 5), and Sera (Table 6)

All animals remained healthy throughout the experimental period. There were no significant differences in the body weights, food and water intake, liver, small intestine, brain, muscle, heart, or kidney weights of mice fed four types of diets. Food intake (g) per day of each group was 24.16 ± 2.14, 24.13 ± 2.63, 23.45 ± 2.53, 23.80 ± 3.99 for control, LO, SPL (1 %), and SPL (2 %)

Table 3 TL level and fatty acid content of liver

	Group			
	Control	LO	SPL (1%)	SPL (3%)
Liver weight (g/100 g BW)	4.67 ± 0.28	4.56 ± 0.24	4.86 ± 0.13	4.99 ± 0.09
TL (mg/g tissue)	56.98 ± 6.42	55.72 ± 4.38	58.21 ± 3.86	57.08 ± 4.98
Fatty acid (μmol/g tissue)				
16:0	32.22 ± 15.83	30.69 ± 5.19	29.38 ± 2.78	29.05 ± 2.56
16:1	4.74 ± 2.73	5.71 ± 1.23	5.83 ± 1.13	5.93 ± 0.79
18:0	12.19 ± 5.86	10.19 ± 0.95	9.73 ± 0.59	9.81 ± 1.07
18:1n-9	36.14 ± 25.34	36.76 ± 13.11	33.69 ± 4.61	27.33 ± 3.39
18:1n-7	5.04 ± 2.87	4.09 ± 0.97	4.87 ± 0.70	3.77 ± 0.67
18:2n-6	15.76 ± 7.93^{b}	$9.79 \pm 1.60^{a,b}$	9.07 ± 0.70^{a}	8.39 ± 1.00^{a}
18:3n-3	0.11 ± 0.06^{a}	1.66 ± 0.38^{c}	0.64 ± 0.09^{b}	1.79 ± 0.27^{c}
20:3n-6	2.08 ± 1.04^{b}	$1.30 \pm 0.32^{a,b}$	$1.41 \pm 0.10^{a,b}$	1.13 ± 0.12^{a}
20:4n-6	14.16 ± 7.08^{b}	6.51 ± 0.57^{a}	7.80 ± 0.73^{a}	5.68 ± 0.70^{a}
20:5n-3	0.07 ± 0.07^{a}	2.30 ± 0.45^{c}	1.19 ± 0.21^{b}	3.28 ± 0.32^{d}
22:5n-3	0.09 ± 0.10^{a}	0.98 ± 0.27^{c}	0.58 ± 0.12^{b}	1.26 ± 0.15^{d}
22:6n-3	4.29 ± 2.04^{a}	8.62 ± 2.52^{b}	$7.02 \pm 0.85^{a,b}$	7.96 ± 1.17^{b}
Total saturated	44.31 ± 21.81	41.83 ± 6.18	39.96 ± 3.16	39.70 ± 3.65
Total monounsaturated	47.04 ± 31.66	47.47 ± 15.24	45.26 ± 6.35	37.66 ± 4.69
Total n-6	32.81 ± 16.29^{b}	18.02 ± 2.53^{a}	18.67 ± 1.49^{a}	15.50 ± 1.83^{a}
Total n-3	4.57 ± 2.08^{a}	13.88 ± 2.88^{c}	9.48 ± 1.14^{b}	14.47 ± 1.86^{c}

Different letters show significantly different at $P < 0.05$

Table 4 TL level and fatty acid content of small intestine

	Group			
	Control	LO	SPL (1 %)	SPL (3 %)
Small intestine weight (g/100 g BW)	3.32 ± 0.28	3.46 ± 0.16	3.00 ± 0.07	3.43 ± 0.16
TL (mg/g tissue)	23.97 ± 5.49	17.07 ± 2.53	23.87 ± 4.55	23.18 ± 5.59
Fatty acid (μmol/g tissue)				
16:0	9.61 ± 5.32	8.06 ± 1.74	10.24 ± 3.31	9.53 ± 5.17
18:0	3.02 ± 1.06	2.71 ± 0.58	2.62 ± 0.81	2.06 ± 0.95
18:1n-9 + 18:1n-7	15.30 ± 9.43	12.73 ± 2.60	18.29 ± 6.61	14.83 ± 8.35
18:2n-6	4.68 ± 2.21^{b}	$2.72 \pm 0.36^{a,b}$	$2.79 \pm 0.88^{a,b}$	2.36 ± 1.23^{a}
18:3n-3	0.09 ± 0.05^{a}	$0.77 \pm 0.16^{b,c}$	$0.43 \pm 0.13^{a,b}$	1.04 ± 0.57^{c}
20:4n-6	0.91 ± 0.27^{b}	$0.58 \pm 0.14^{a,b}$	0.56 ± 0.26^{a}	0.37 ± 0.19^{a}
20:5n-3	0.00 ± 0.00^{a}	0.17 ± 0.05^{c}	$0.08 \pm 0.04^{a,b}$	$0.15 \pm 0.08^{b,c}$
22:5n-3	0.02 ± 0.01^{a}	0.14 ± 0.03^{b}	$0.08 \pm 0.04^{a,b}$	0.16 ± 0.08^{b}
22:6n-3	0.28 ± 0.11^{a}	0.51 ± 0.10^{b}	$0.39 \pm 0.16^{a,b}$	$0.35 \pm 0.18^{a,b}$
Saturated	13.21 ± 6.54	11.25 ± 2.19	13.49 ± 4.18	12.18 ± 6.37
Monounsaturated	18.31 ± 11.36	15.46 ± 3.27	22.26 ± 8.08	18.64 ± 10.64
Total n-6	5.80 ± 2.28^{b}	$3.48 \pm 0.46^{a,b}$	$3.55 \pm 1.17^{a,b}$	2.85 ± 1.45^{a}
Total n-3	0.38 ± 0.14^{a}	1.59 ± 0.18^{b}	$0.98 \pm 0.32^{a,b}$	1.70 ± 0.87^{b}

Different letters show significantly different at $P < 0.05$

Table 5 Fatty acid composition of serum (wt% of total fatty acids)

	Group			
	Control	LO	SPL (1 %)	SPL (3 %)
Fatty acid				
16:0	24.82	25.24	24.36	25.60
16:1	3.11	2.95	3.42	3.70
18:0	10.68	12.19	11.48	10.95
18:1n-9	23.50	23.81	26.56	22.97
18:1n-7	3.42	2.77	3.58	2.93
18:2n-6	16.78	13.45	12.40	13.22
18:3n-6	0.16	0.08	0.09	0.09
18:3n-3	0.09	1.13	0.66	1.86
18:4n-3	ND	0.04	0.02	0.04
20:3n-6	1.89	1.46	1.54	1.26
20:4n-6	7.41	3.60	4.14	3.31
20:5n-3	0.22	1.71	0.84	2.52
22:5n-3	0.09	0.68	0.42	1.01
22:6n-3	2.63	6.00	4.63	5.75
Saturated				
Monounsaturated				
Total n-6	26.24	18.59	18.17	17.88
Total n-3	3.03	9.56	6.57	11.18

ND not detected

group, respectively. There was also no significant difference in dairy food intake among different four groups. Supplementation of ALA-rich diets, including LO, SPL (1 %), and SPL (3 %), significantly increased hepatic n-3 PUFA levels, including ALA, EPA, and docosapentaenoic acid (DPA, 22:5n-3), compared with the control, while a significant decrease in n-6 arachidonic acid (ARA, 20:4n-6) was found in the ALA-rich diet feedings (Table 3). The LA level of mice fed ALA-rich diets also decreased, although the LA content in the ALA diets was higher than that in the control (Table 2). Mice fed LO and SPL (3 %) showed significantly higher DHA levels relative to the control. The DHA level in mice fed SPL (1 %) also increased, but the difference compared with the control was not significant.

Although the ALA content of the diet was the same for LO and SPL (3 %) (Table 2), significantly higher contents of hepatic EPA and DPA were found in mice fed SPL (3 %) than in the LO group (Table 3). Additionally, the ALA level of the mice fed SPL (3 %) was higher than those fed LO, but the difference was not significant. The higher ALA level in the mice fed SPL (3 %) than those fed LO was also found in the small intestinal lipids (Table 4) and in the serum lipids (Table 5). In serum lipids, EPA and DPA were also much higher in mice fed SPL (3 %) than in the LO group. However, the difference in the fatty acid contents in brain lipids was small (Table 6).

Although there were no significant differences in serum total cholesterol (Fig. 1a), LDL cholesterol (Fig. 1c), neutral lipids (Fig. 1d), PL (Fig. 1e), and free fatty acid (FFA) (Fig. 1f) levels among the four dietary groups, HDL cholesterol (Fig. 1b) significantly increased in mice fed SPL (1 %) and SPL (3 %).

Table 6 TL level and fatty acid content of brain

	Group			
	Control	LO	SPL (1 %)	SPL (3 %)
Brain weight (g/100 g BW)	1.63 ± 0.05	1.62 ± 0.04	1.66 ± 0.04	1.63 ± 0.03
TL (mg/g tissue)	69.01 ± 2.40	67.81 ± 3.34	64.79 ± 2.49	71.66 ± 1.41
Fatty acid (μmol/g tissue)				
16:0	21.78 ± 1.97	21.06 ± 3.19	21.43 ± 2.40	23.39 ± 1.29
18:0	17.23 ± 1.85	16.20 ± 3.12	16.68 ± 1.75	18.38 ± 0.81
18:1n-9	15.60 ± 1.90	15.62 ± 1.65	14.33 ± 1.99	16.42 ± 1.40
18:1n-7	3.66 ± 0.34	3.41 ± 0.42	3.23 ± 0.44	3.59 ± 0.31
18:2n-6	0.47 ± 0.08[b]	0.08 ± 0.05[b]	0.32 ± 0.05[a]	0.38 ± 0.01[a,b]
20:3n-6	0.37 ± 0.08	0.44 ± 0.04	0.39 ± 0.08	0.46 ± 0.06
20:4n-6	7.44 ± 0.99	6.68 ± 1.49	6.97 ± 0.73	7.33 ± 0.85
20:5n-3	ND	0.02 ± 0.04	0.02 ± 0.05	0.04 ± 0.06
22:5n-3	ND[a]	0.12 ± 0.12[a,b]	0.05 ± 0.10[a,b]	0.18 ± 0.11[b]
22:6n-3	10.60 ± 1.61	11.09 ± 2.02	10.82 ± 1.18	12.04 ± 1.00
Saturated	39.49 ± 3.78	37.70 ± 6.06	38.52 ± 4.06	42.21 ± 1.96
Monounsaturated	19.26 ± 2.23	19.03 ± 2.04	17.57 ± 2.43	20.02 ± 1.70
Total n-6	8.34 ± 1.13	7.63 ± 1.54	7.73 ± 0.83	8.23 ± 0.88
Total n-3	10.60 ± 1.61	11.23 ± 2.02	10.89 ± 1.26	12.26 ± 1.10

Different letters show significantly different at $P < 0.05$

ND not detected

Gene Expression of Elongase, Desaturase, and Peroxisomal Enzymes Involved in ALA Bioconversion to DHA

ALA is converted to DHA through a series of desaturation and chain elongation processes. Figure 2 shows the effect of the experimental diets on the relative mRNA expression levels of these fatty acids desaturase, elongase, and acyl CoA oxidase in the liver. SPL (3 %) supplementation significantly decreased Fads1 and Fads2, while a significant increase in Acox1 was found in the SPL (3 %) group. However, LO had no significant effect on these gene expressions compared with the control group.

Discussion

Supplementation of ALA-rich lipids, LO, and SPL resulted in increased ALA and its n-3 metabolites (EPA, DPA, and DHA) and decreased n-6 PUFA (LA and ARA) in mice livers, small intestines, and sera (Tables 3, 4, 5). Increased n-3 PUFA and decreased n-6 PUFA in rat organs, including the brain, as a result of ALA feeding have been reported in other studies [32–34]; however, in the present study, little effect on the fatty acid composition of brain lipids was observed (Table 6). Lipid and fatty acid compositions of the brain are usually less affected by dietary lipids in normal conditions and strictly regulated through DHA uptake from the plasma and brain DHA metabolism [18]. Therefore, the result in Table 6 may be due to homeostasis found in normal C57BL/6J mice administered the experimental diet for only 4 weeks in the present study. The same result has also been obtained in female ddy mice fed DHA-rich lipids [26]. They reported that the DHA levels of the serum and the liver lipids were significantly increased by feeding DHA lipids, but the fatty acid composition of the brain did not change drastically.

Although the ALA contents in the LO and SPL (3 %) diets were the same (Table 2), the EPA and DPA levels in the liver lipids from the mice fed the SPL (3 %) diet were significantly higher than those fed the LO diet (Tables 3, 5). Additionally, the ALA level of lipids from the livers, small intestines, brains, and plasma of mice fed the SPL (3 %) diet were also higher than those fed the LO diet (Tables 3, 4, 5). These results suggest that ALA originated from SPL may be absorbed more efficiently than that from LO. However, a significant increase in EPA and DPA levels of hepatic lipids from mice fed SPL (3 %), shown in Table 2, may be derived from up-regulation of ALA bioconversion to EPA and DPA. This reaction is regulated by the activities of different enzymes, including Δ^6-desaturase (Fads2), elongase-5 (Elov5), Δ^5-desaturase (Fads1), and elongase-2 (Elov2). However, the gene expressions of Fads2 and Fads1 significantly decreased in the SPL fed group (Fig. 2). Additionally, SPL feeding had no significant effect on both elongases, showing little effect of SPL feeding on up-regulating bioconversion of ALA to EPA and DPA.

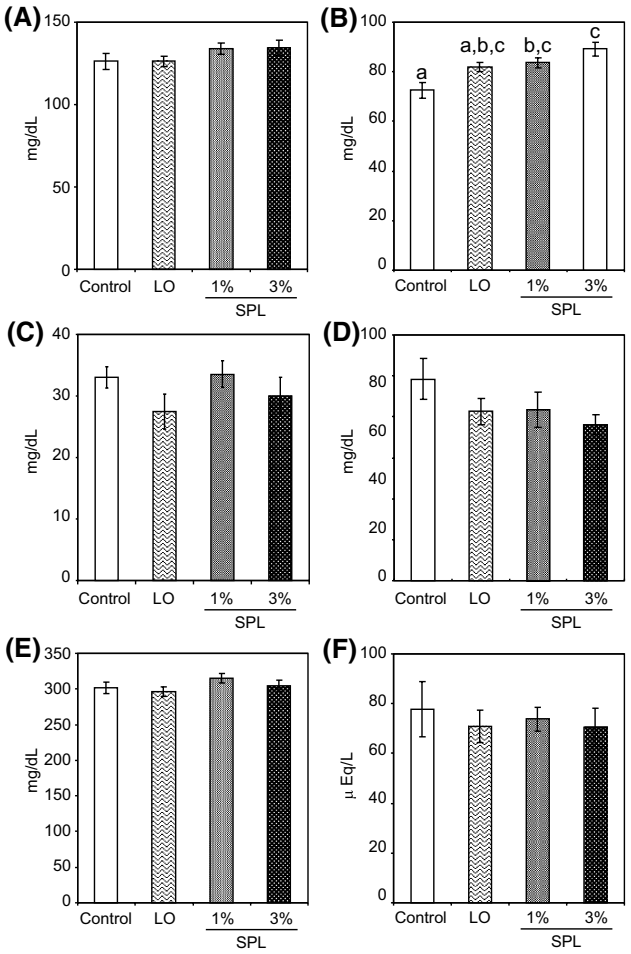

Fig. 1 Effects of dietary lipids on serum lipid parameters of C57BL/6 J mice. **a** Total cholesterol; **b** HDL cholesterol; **c** LDL cholesterol; **d** neutral lipids; **e** phospholipids (PL); **f** free fatty acids (FFA). Values represent mean ± SE of seven mice per group. *Different letters* show significant differences at $P < 0.05$

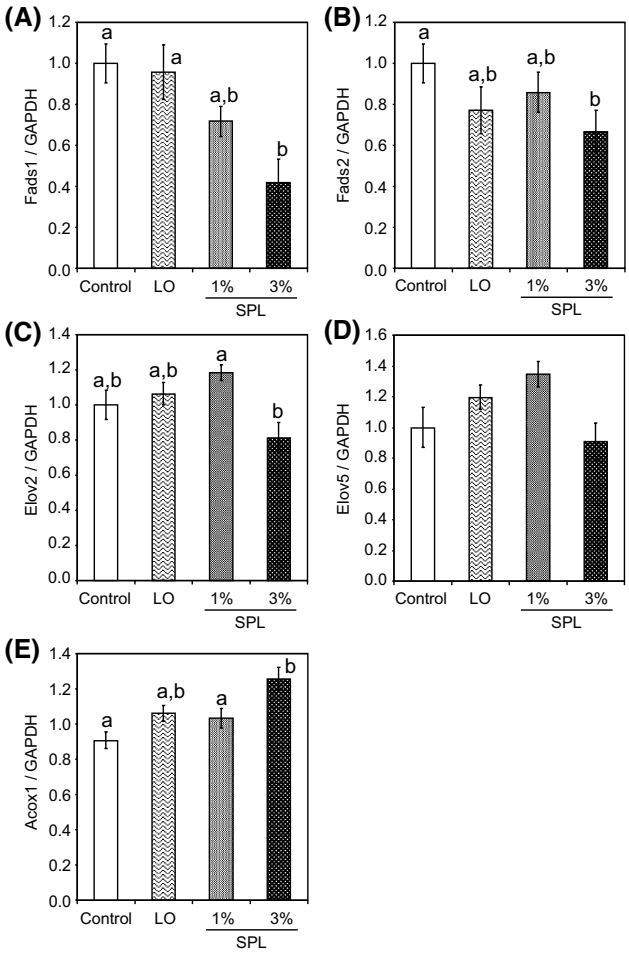

Fig. 2 Expression of mRNA of genes associated with the bioconversion of α-linolenic acid (ALA) to docosahexaenoic acid (DHA) in C57BL/6J mice fed different dietary lipids. Values represent mean ± SE of seven mice per group. *Different letters* show significant differences at $P < 0.05$

In LO, greater than 99 % of ALA was present as TAG, whereas most ALA in SPL was incorporated into GLs, such as MGDG or DGDG [3, 5–8]. We have reported 75.2 and 77.2 % ALA in MGDG and DGDG from spinach powder lipids, respectively [35]. Both GL were the major lipid components of SPL, and a high ALA level (53.2 %) was found in the SPL used in the present study. The higher levels of ALA and of its metabolites, EPA and DPA, in the mice fed the SPL (3 %) diet was probably due to the higher absorption rate of ALA in the MGDG and/or DGDG forms from an intestinal part.

Several studies have demonstrated the absorption efficacy of n-3 PUFA from different chemical forms [25, 36]. Generally, PL has been considered to be better absorbed than the TAG form, especially in infants [25]. A comparative study using free fatty acids (FFA), EE, TAG, and PL forms of DHA showed that DHA-PL was more effective at increasing DHA in the liver and the brain of male Balb/c mice than other DHA forms [27]. A human study on the uptake of n-3 PUFA as PL form has been reported using krill oil containing 30–65 % of the fatty acids as the PL form. In a double-blinded crossover trial, Schuchardt et al. [24] compared the uptake of EPA + DHA from three different oral administrations of fish oil TAG, EE, and krill oil (mainly PL). Although the intake levels of EPA and DHA were the same among the three groups, the krill oil group showed the highest incorporation of EPA + DHA into plasma, followed by TAG and EE.

The amphiphilic character of PL has been proposed as the most likely reason for the higher intake of EPA + DHA from PL compared with that from TAG [24]. ALA bound to GL is also due to the amphiphilic character of this lipid, similarly to PL. GL possesses emulsification properties due to the presence of a galactosyl group. As a result, GL

has been shown to influence the surface composition of fat droplets and increase the binding rate of hydrolyzing enzymes [37]. This surfactant ability of GL has also been shown to promote the formation of mixed micelles and, therefore, the digestion. The first step in GL digestion is hydrolysis of the *sn*-1 position on the glycerol backbone by pancreatic lipase, yielding monoacylgalactolipids [22]. This lyso compound shows higher emulsification properties than the corresponding diacylglycerols from TAG. Thus, it may be possible that the high GL level in SPL leads to an enhanced absorption of lipids rich in ALA. However, the present study only analyzed fatty acid distribution of several tissues and sera. This study has a small sample size and was short in duration (only 4 weeks). In addition, there were no measurements of n-3 PUFA excretion and no direct measurement of n-3 PUFA intake. While we did measure fatty acids in serum, liver, brain, and intestine, several tissues were not examined, including the heart and skeletal muscle, that can accumulate and serve as deposits of n-3 PUFA [38]. Longer experiments in different types of animals to ensure fatty acid levels reach equilibrium and a comprehensive fatty acid analysis of blood lipids (plasma, erythrocytes, or leukocytes) and other tissues are needed. For further insight on absorption and metabolism of n-3 PUFA from glycolipids, intake, and excretion measurements as well as tracer studies using isotope-labeled ALA are needed [39–41].

Numerous epidemiological studies, clinical trials, genetic, and nutrigenetic approaches have demonstrated the health benefits of n-3 PUFA such as ALA, EPA, and DHA. The most apparent benefit of these n-3 PUFA is reduction of cardiovascular risk, probably due to regulation of membrane structure, lipid metabolism, blood clotting, blood pressure, and inflammation [42]. Dietary ALA has been known to improve blood lipid levels [9, 19]. However, there was no significant effect of dietary ALA on plasma total cholesterol, LDL cholesterol, neutral lipids, PL, and FFA levels found in the present study using normal mice fed normal diets, although a decreasing trend in neutral lipids, FFA, and LDL cholesterol was observed in ALA-containing diets (Fig. 1). However, significant increases in HDL cholesterol were found in the mice fed SPL (1 %) and (3 %) diets (Fig. 1). Although the reason for the higher HDL levels is unknown, the effect of SPL should be attributed to the biological activities of the characteristic lipid constituents of SPL containing MGDG, DGDG, and SQDG as major components. Studies on both synthetic and natural MGDG and DGDG have revealed their activities, including anti-tumor, anti-inflammatory, and cell cycle regulation [43–45]; however, no studies regarding the effect of GL on lipid metabolism have been conducted. SPL also contained lutein as a major carotenoid. Lutein has attracted great attention for preventing and reversing certain serious eye diseases [46, 47]; however, little is known about the effect of lutein on lipid metabolism. More studies may need to be conducted on the biological effects of these leafy lipid components.

In conclusion, the present study demonstrated the important role of green leafy vegetables as n-3 sources. Leafy vegetable lipids, such as SPL, are mainly composed of DGDG and MGDG rich in ALA. A wide range of plant products contain ALA, including seeds, nuts, vegetables, legumes, grains, and fruits. Among them, several types of seed oils, such as LO, flaxseed oil, and walnut oil, are known to be ALA sources. In addition to these seed oils, green leafy vegetable lipids should also be considered because they are rich in ALA as GL forms and in phytochemicals, such as carotenoids.

Acknowledgments This work was supported by the "Scientific technique research promotion program from agriculture, forestry, fisheries, and food industry" from the Ministry of Agriculture, Forestry, and Fisheries in Japan.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

1. Harwood JL (1979) The synthesis of acyl lipids in plant tissues. Prog Lipid Res 18:55–86
2. Kamogawa H, Hosokawa M, Abe M, Miyashita K (2012) Carotenoid contents in *Chlorella pyrenoidosa* and concentration by saponification. Bull Fish Sci Hokkaido Univ 62:83–88 **(Japanese)**
3. Harwood JL (1980) Plant acyl lipids: structure, distribution and analysis. In: Stump PK, Conn EE (eds) Biochemistry of plants, vol 4. Academic Press, New York, pp 1–55
4. Block MA, Dorne AJ, Joyard J, Douce R (1983) Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. J Biol Chem 258:13281–13286
5. Whitaker BD (1986) Fatty-acid composition of polar lipids in fruit and leaf chloroplasts of "16:3"—and "18:3"—plant species. Planta 169:313–319
6. O'Brien JS, Benson AA (1964) Isolation and fatty acid composition of the plant sulfolipid and galactolipids. J Lipid Res 5:432–436
7. Bahl J, Francke B, Monéger R (1976) Lipid composition of envelopes, prolamellar bodies and other plastid membranes in etiolated, green and greening wheat leaves. Planta 129:193–201
8. Nichols BW (1965) The lipids of moss (*Hypnum cupressiforme*) and of the leaves of green holly (*Ilex aquifolium*). Phytochemistry 4:769–772
9. Kim K-B, Nam YA, Kim HS, Hayes AW, Lee B-M (2014) α-Linolenic acid: nutraceutical, pharmacological and toxicological evaluation. Food Chem Toxicol 70:163–178
10. Allayee H, Roth N, Hodis HN (2009) Polyunsaturated fatty acids and cardiovascular disease: implications for nutrigenetics. J Nutrigenet Nutrigenomics 2:140–148

11. Harris WS, Miller M, Tighe AP, Davidson MH, Schaefer EJ (2008) Omega-3 fatty acids and coronary heart disease risk: clinical and mechanistic perspectives. Atherosclerosis 197:12–24
12. Mori TA (2014) Dietary n-3 PUFA and CVD: a review of the evidence. Proc Nutr Soc 73:57–64
13. Superko HR, Superko AR, Lundberg GP, Margolis B, Garrett BC, Nasir K, Agatston AS (2014) Omega-3 fatty acid blood levels clinical significance update. Curr Cardiovasc Risk Rep 8:407
14. Wachira JK, Larson MK, Harris WS (2014) n-3 Fatty acids affect haemostasis but do not increase the risk of bleeding: clinical observations and mechanistic insights. Br J Nutr 111:1652–1662
15. Carnielli VP, Simonato M, Verlato G, Luijendijk I, De Curtis M, Sauer PJJ, Cogo PE (2007) Synthesis of long-chain polyunsaturated fatty acids in preterm newborns fed formula with long-chain polyunsaturated fatty acids. Am J Clin Nutr 86:1323–1330
16. Williams CM, Burdge G (2006) Long-chain n-3 PUFA: plant v. marine sources. Proc Nutr Soc 65:42–50
17. Brenna JT, Salem N Jr, Sinclair AJ, Cunnane SC (2009) α-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. Prostaglandins Leukot Essent Fatty Acids 80:85–91
18. Domenichiello AF, Chen CT, Trepanier M-T, Stavro PM, Bazinet RP (2014) Whole body synthesis rates of DHA from α-linolenic acid are greater than brain DHA accretion and uptake rates in adult rats. J Lipid Res 55:62–74
19. Barceló-Coblijn G, Murphy EJ (2009) Alpha-linolenic acid and its conversion to longer chain n-3 fatty acids: benefits for human health and a role in maintaining tissue n-3 fatty acid levels. Prog Lipid Res 48:355–374
20. Kris-Etherton PM, Harris WS, Appel LJ (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation 106:2747–2757
21. Andersson L, Bratt C, Arnoldsson KC, Herslöf B, Olsson NU, Sternby B, Nilsson Å (1995) Hydrolysis of galactolipids by human pancreatic lipolytic enzymes and duodenal contents. J Lipid Res 36:1392–1400
22. Sugawara T, Miyazawa T (2000) Digestion of plant monogalactosyldiacylglycerol and digalactosyldiacylglycerol in rat alimentary canal. J Nutr Biochem 11:147–152
23. Dyerberg J, Madsen P, Møller JM, Aardestrup I, Schmidt EB (2010) Bioavailability of marine n-3 fatty acid formulations. Prostaglandins Leukot Essent Fatty Acids 83:137–141
24. 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
25. Ramíreza M, Amatea L, Gil A (2001) Absorption and distribution of dietary fatty acids from different sources. Early Hum Dev 65:S95–S101
26. Tanaka Y, Ohkubo T, Fukuda N, Hibino H (2003) Effect of molecular forms on distribution of docosahexaenoic acid into organs in mice. J Oleo Sci 52:89–97
27. Tang X, Li Z-J, Xu J, Xue Y, Li J-Z, Wang J-F, Yanagita T, Xue C-H, Wang Y-M (2012) Short term effects of different omega-3 fatty acid formulation on lipid metabolism in mice fed high or low fat diet. Lipids Health Dis 11:70
28. Ulven SM, Kirkhus B, Lamglait A, Basu S, Elind E, Haider T, Berge K, Vik H, Pedersen JI (2011) Metabolic effects of krill oil are essentially similar to those of fish oil but at lower dose of EPA and DHA, in healthy volunteers. Lipids 46:37–46
29. Prevot AF, Mordret FX (1976) Utilisation des colonnes capillaries de verre pour l'analyse des corps gras par chromotographie en phase gazeuse. Rev Fse Corps Gras 23:409–423
30. Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509
31. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
32. Blanchard H, Pédrono F, Boulier-Monthéan N, Catheline D, Rioux V, Legrand P (2013) Comparative effects of well-balanced diets enriched in α-linolenic or linoleic acids on LC-PUFA metabolism in rat tissues. Prostaglandins Leukot Essent Fatty Acids 88:383–389
33. Delplanque B, Du Q, Agnani G, LeRuyet P, Martin JC (2013) A dairy fat matrix providing alpha-linolenic acid (ALA) is better than a vegetable fat mixture to increase brain DHA accretion in young rats. Prostaglandins Leukot Essent Fatty Acids 88:115–120
34. Poudyal H, Panchal SK, Ward LC, Brown L (2013) Effects of ALA, EPA and DHA in high-carbohydrate, high-fat diet-induced metabolic syndrome in rats. J Nutr Biochem 24:1041–1052
35. Yamaguchi T, Sugimura R, Shimajiri J, Suda M, Abe M, Hosokawa M, Miyashita K (2012) Oxidative stability of glyceroglycolipids containing polyunsaturated fatty acids. J Oleo Sci 61:505–513
36. Burri L, Hoem N, Banni S, Berge K (2012) Marine omega-3 phospholipids: metabolism and biological activities. Int J Mol Sci 13:15401–15419
37. Mun S, Decker EA, McClements DJ (2007) Influence of emulsifier type on *in vitro* digestibility of lipid droplets by pancreatic lipase. Food Res Int 40:770–781
38. Salem NM, Lin YH, Moriguchi T, Lim SY, Salem N Jr, Hibbeln JR (2015) Distribution of omega-6 and omega-3 polyunsaturated fatty acids in the whole rat body and 25 compartments. Prostaglandins Leukot Essent Fatty Acids 100:13–20
39. Ghasemifard S, Hermon K, Turchini GM, Sinclair AJ (2014) Metabolic fate (absorption, β-oxidation and deposition) of long-chain n-3 fatty acids is affected by sex and by the oil source (krill oil or fish oil) in the rat. Br J Nutr 114:684–692
40. 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
41. Domenichiello AF, Kitson AP, Bazinet RP (2015) Is docosahexaenoic acid synthesis from α-linolenic acid sufficient to supply the adult brain? Prog Lipid Res 59:54–66
42. Guichardant M, Calzada C, Bernoud-Hubac N, Lagarde M, Véricel E (2015) Omega-3 polyunsaturated fatty acids and oxygenated metabolism in atherothrombosis. Biochim Biophys Acta 1851:485–495
43. Bruno A, Rossi C, Marcolongo G, Di Lena A, Venzo A, Berrie CP, Corda D (2005) Selective *in vivo* anti-inflammatory action of the galactolipid monogalactosyldiacylglycerol. Eur J Pharmacol 524:159–168
44. Maeda N, Hada T, Yoshida H, Mizushina Y (2007) Inhibitory effect on replicative DNA polymerases, human cancer cell proliferation, and *in vivo* anti-tumor activity by glycolipids from spinach. Curr Med Chem 14:955–967
45. Maeda N, Kokai Y, Ohtani S, Sahara H, Kumamoto-Yonezawa Y, Kuriyama I, Hada T, Sato N, Yoshida H, Mizushina Y (2008) Anti-tumor effect of orally administered spinach glycolipid fraction on implanted cancer cells, colon-26, in mice. Lipids 43:741–748
46. Ma L, Lin X-M (2010) Effects of lutein and zeaxanthin on aspects of eye health. J Sci Food Agric 90:2–12
47. Ruban AV, Johnson MP (2010) Xanthophylls as modulators of membrane protein function. Arch Biochem Biophys 504:78–85