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Isolation and Characterization of Neutral Lipids of Desilked Eri Silkworm Pupae Grown on Castor and Tapioca Leaves

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The neutral lipid of desilked eri silkworm pupae (*Samia cynthia ricini*) grown on two different host plants, castor (*Ricinus communis Linn.*) and tapioca (*Manihot utilizsima Phol.*) leaves, was extracted with hexane. The oil content in pupae was estimated to be in the range of 18−20% (dry basis). The pupal oil was found to be enriched with α-linolenic acid (ALA) with palmitic acid as the second major fatty acid. The level of ALA in the oil of silkworm pupae was found to be significantly higher ($P < 0.001$) when grown on tapioca (58.3%) as compared to those grown on castor (42.9%). Other chemical parameters such as percent free fatty acid, peroxide value, phosphorus content, percent unsaponifiable matter, and composition of sterols were also determined in both of the oils and compared. Reversed-phase high-performance liquid chromatography analysis of triacylglycerol molecular species showed that the pupal oil is rich in molecular species with equivalent carbon numbers (ECN) C36, C40, C42, C44, and C48. There was a significantly higher level ($P < 0.001$) of trilinolenin (C36) in the oil of tapioca-based silkworm as compared to castor-based silkworm pupae. Regiospecific analysis of the oil showed a higher level of ALA at the *sn*-2 position of silkworm pupae grown on tapioca (60.2%) as compared to those grown on castor (47.3%) oil. Thus, the presence of a large amount of ALA and their predominance at the *sn*-2 position make the eri pupal oil highly nutritious, provided that the oxidative stability is ensured.

KEYWORDS: Eri silkworm; *Samia cynthia ricini*; pupae; neutral lipid; triacylglycerol; castor leaf; tapioca leaf; RP-HPLC

INTRODUCTION

Eri silk is an important variety of nonmulberry silk known for its elegance and is produced primarily in the northeastern states of India. Almost 98% of a total of 1300 tons of eri silk produced in the country comes from the northeastern states. The word "eri" originates from the word "eranda", meaning castor (*Ricinus communis Linn.*), the most important host plant of the eri silkworm. Other host plants for eri silkworm are tapioca (*Manihot utilizsima Phol.*), kesseru (*Heteropanax fragrans Seem.*), payam (*Evodia flaxinifolia*), barpat (*Ailenthus grandis Roxb.*), and barkesseru (*Ailenthus excelsa Roxb.*) (*1*). Silkworms, being phytophagus insects, show associations with host plants on which they are grown. These host plants provide the necessary nutrients required by the insect for various metabolic activities and also for silk production (*2*).

Ericulture, i.e., the rearing of silkworms, *Samia cynthia ricini* (Donovan), has been introduced in many states in India outside the northeastern states. The byproducts, desilked pupae, are known for their nutritional value due to the presence of high protein and high fat (*3*) and are a delicacy among the tribal populations. Even then, the consumption of spent silkworm pupae is not popular among the general population. The neutral lipid of silkworm pupae was considered to be a good source of α-linolenic acid (ALA). This is based on numerous literature reports on fatty acid composition of neutral lipids predominantly of silkworm pupae, *Bombyx mori* L. (*4*−*7*), although there were variations in the level of ALA (traces to 40% of total fatty acids) reported so far in the *B. mori* L. silkworm pupae. Eri silkworm has not been explored so far in characterizing the composition of neutral lipids, and there is no literature report on such studies.

The objective of the present study is value addition to spent silkworm pupae, a major portion of which otherwise are either thrown away as sericulture waste or used as a fertilizer and as a constituent of chick and fish feeds (*4*, *8*). The desilked eri pupae were collected from the local silk industry for the extraction of oil. The extracted oil was characterized and analyzed for the estimation of free fatty acids, peroxide value, phosphorus content, unsaponifiable matter, composition of fatty acids, composition of triacylglycerol (TAG) molecular species, positional distribution of fatty acids over the glycerol backbone, and composition of sterols. A comparative study was also

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conducted to find the influence of host plants on all of these studied parameters of oil extracted from eri pupae grown on two different host plants, namely, castor and tapioca.

MATERIALS AND METHODS

Chemicals. Reference TAG (catalog no. 17811), fatty acid mixtures (catalog no. 18919), silylating agent, N,O-bis(trimethylsilyl)trifluoroacetamide, BSTFA (catalog no. T1506-25G), and diazomethane precursor, *N*-methyl-*N*-nitroso-*p*-toluene sulfonamide (catalog no. M2256), were purchased from Sigma Chemical Co. (United States). A standard plant sterols kit (catalog no. 4-7256) was purchased from Supelco Inc. (United States). Precoated thin-layer chromatography (TLC) plates (silica gel 60 F_{254}) were purchased from Merck (Germany). All other analytical reagent grade chemicals and solvents were purchased from a local market.

Extraction of Pupal Oil. Matured pupae (1 kg) grown on castor (Nalgonda, Andhra Pradesh, India) and tapioca (Rampachodavarm, Andhra Pradesh, India) leaves were collected and kept in a steam oven at 80−90 °C under a vacuum of 250−260 mm Hg for 8−10 h to get a constant weight (262 g for castor and 275 g for tapioca-based silkworms). The water content in pupae was found to be in the range of 72−74%. Roasted pupae were finely ground to a powder for Soxhlet extraction with hexane for 8−10 h and evaporated to get pupal oil (53.4 g for castor and 49.6 g for tapioca-based silkworm).

Characterization of Pupal Oil. The free fatty acid percent (*9*), peroxide value (*10*), and unsaponifiable matter content (*11*) were determined following Official Methods of the AOCS, and the phosphorus content was determined by an IUPAC method (*12*). The phospholipids in the oil were estimated from phosphorus content as described in Official Methods of the AOCS (*13*). The composition of sterol was determined according to Official Methods of the AOCS (*14*). The sterol mixture was separated from unsaponifiable matter by TLC using $CHCl_3$ as a developing solvent and transformed into their trimethylsilyl ethers using the silylating agent BSTFA (*15*). For identification of individual components present in sterols, silylated sterols were injected in gas chromatography−mass spectrometry (GC-MS). The GC-MS detection was performed with an Agilent 6890N Gas Chromatograph connected to an Agilent 5973 Mass Selective Detector at 70 eV (*m*/*z* 50−550; source at 230 °C and quadruple at 150 °C) in the electron impact mode with a HP-5 ms capillary column (30 m × 0.25 mm i.d. × 0.25 μM film thickness). The oven temperature was programmed for 2 min at 200 °C to 300 °C at 4 °C/min and maintained for 20 min at 300 °C. The carrier gas, helium, was used at a flow rate of 1.0 mL/min. The inlet temp was maintained at 300 °C, and the split ratio was 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation patterns of authentic compounds. For quantification, silylated sterols were analyzed using an Agilent 6890 Series Gas Chromatograph equipped with a flame ionization detector (FID) and the capillary column HP-1 (30 m × 0.25 mm i.d. × 0.5 μm film thickness). The injector and detector temperatures were maintained at 300 and 325 °C, respectively. The oven temperature was programmed for 2 min at 200 °C to 300 °C at 6 °C/min and maintained for 20 min at 300 °C. The carrier gas, nitrogen, was used at a flow rate of 1.5 mL/min. The injection volume was 1 μL, with a split ratio of 50:1. Sterol quantification was achieved by use of 1% chloroformic solution of α-cholestanol as an internal standard added into the unsaponifiable matter before purification.

Fatty Acid Composition of Pupal Oil. Pupal oil was converted to its fatty acid methyl esters (FAME) according to the method described by Christie (*16*) using 2% H_2SO_4 in methanol as a methylating agent. The resulting FAME was injected in GC for compositional analysis. GC was performed on an Agilent 6890 Series Gas Chromatograph equipped with a FID and the capillary column DB-23 (30 m × 0.25 mm i.d. × 0.5 μm film thickness). The injector and detector temperatures were maintained at 230 and 250 °C, respectively. The oven temperature was programmed for 2 min at 160 °C to 180 °C at 6 °C/min, maintained for 2 min at 180 °C, increased further to 230 °C at 4 °C/min, and finally maintained for 10 min at 230 °C. The carrier gas, nitrogen, was used at a flow rate of 1.5 mL/min. The injection volume was 1 μL, with a split ratio of 50:1.

Table 1. Comparative Characteristic of Oils Extracted from Silkworm Pupae Grown on Castor and Tapioca Leaves[a]

characteristics	pupal oil	
	castor fed	tapioca fed
free fatty acid (%)	1.0 ± 0.25	1.9 ± 0.10
peroxide value (ppm)	2.9 ± 0.50	1.7 ± 0.20[b]
phosphorus content (ppm)	1005 ± 31.9	1180 ± 38.5[c]
unsaponifiable matter (%)	2.1 ± 0.10	2.2 ± 0.11

[a] Results are expressed as means ± SD for three samples. [b] Significantly different from castor group: $P < 0.02$. [c] Significantly different from castor group: $P < 0.01$.

TAG Molecular Species Composition of Pupal Oil. The reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was performed on an HP-1050 series HPLC equipped with an ELSD 2000 (Alltech Associates Inc., United States). About 25 μL of extracted oil (1 mg/mL) was injected in the SGE RP-column (250 SS 4.6-W5C18-RS). The molecular species of TAGs were eluted within 10 min using an isocratic mobile phase of 95:5 (v/v) of acetone/2-propanol at a flow rate of 1 mL/min. The molecular species of pupal oil were identified by their equivalent carbon numbers (ECN), by injecting reference TAG mixtures and also by comparing with the literature data (*17*, *18*). The operating conditions for ELSD are as follows: drift tube temperature, 30 °C; flow of nitrogen, 1.5 L/min with impactor "on" mode.

Leaf Total Lipid Fatty Acid Composition. Fresh castor and tapioca leaves were collected, cleaned, and air-dried. The total lipids were extracted from the leaves in a 2:1 ratio (v/v) of chloroform/methanol using the Folch method (*19*). The extract was filtered, evaporated to complete dryness, and converted to FAME (*16*). The reaction mixture was purified by prep-TLC using 90:10 (v/v) of hexane/ethyl acetate as the developing solvent. The FAME band was scrapped out, eluted with ethyl acetate, and injected in GC for compositional analysis. The gas chromatographic conditions were the same as those applied during analysis of pupal oil fatty acid composition.

Regiospecific Analysis of Pupal Oil. Regiospecific analysis of TAGs of the extracted oil from eri pupae was subjected to porcine pancreatic lipase hydrolysis as described by Christie (*20*). Hydrolyzed components were separated by prep-TLC using 70:30:2 (v/v/v) of hexane/ethyl acetate/acetic acid as the developing solvent, and the bands corresponding to 2-monoacylglycerol (MAG) and free fatty acids (FFA) were scrapped out. The MAG was transmethylated to FAME by treatment with 2% H_2SO_4 in methanol (*16*) and injected in GC to determine the distribution of fatty acids at the *sn*-2 position. The released FFA was converted to FAME by treatment with diazomethane (*16*) and injected in GC to find its distribution together at the *sn*-1,3 positions.

Statistical Analysis. The reported results are the means of three measurements, presented as means ± standard deviations (SDs) and were analyzed by a paired Student's *t*-test to evaluate the level of statistical significance. Differences were assessed by one-way analysis of variance. A *P* value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Eri silkworm pupae were found to contain 18−20% of oil (dry basis). The extracted oil was analyzed for different chemical parameters (**Table 1**). The peroxide value is comparatively high ($P < 0.02$) in oil extracted from silkworm pupae when grown on castor leaves as compared to tapioca leaves. The pupal oil also showed quite a high content of phosphorus and, hence, phospholipids (3−3.5%) (*13*). The content is significantly higher ($P < 0.01$) in tapioca-based pupal oil. The free fatty acid contents in oil are comparable between the two studied groups. Although host plant-dependent differences are not observed in the contents of unsaponifiable matter, differences were observed in the level of individual sterol components. The quantitative compositional analysis of sterol was determined and is given in **Table 2**. Among sterols, cholesterol is the major component

Table 2. Sterol Composition (mg/100 g) of Oil Extracted from Silkworm Pupae Grown on Castor and Tapioca Leaves[a]

	pupal oil	
sterol	castor fed	tapioca fed
cholesterol	89.26 ± 0.30	59.26 ± 0.63[b]
campesterol	6.66 ± 0.20	6.0 ± 1.30
stigmasterol	4.53 ± 0.23	
β-sitosterol	36.5 ± 0.10	30.1 ± 6.10
total	136.95 ± 0.55	95.36 ± 7.39[b]

[a] Data are means ± SD for three samples. [b] Significantly different from castor group: $P < 0.001$.

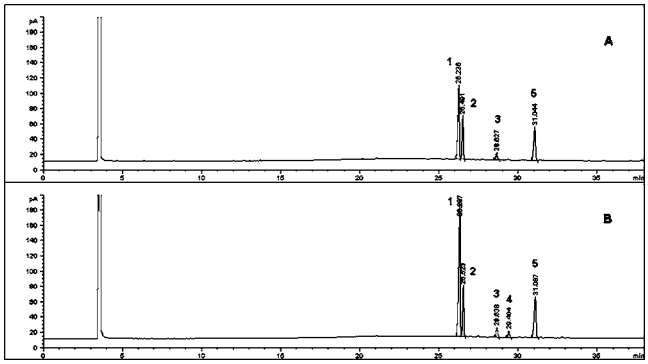

Figure 1. GC chromatogram of a sterol fraction of pupal oil extracted from eri silkworm grown on (**A**) tapioca and (**B**) castor leaves. Peaks: 1, cholesterol; 2, α-cholestanol as internal standard; 3, campesterol; 4, stigmasterol; and 5, β-sitosterol.

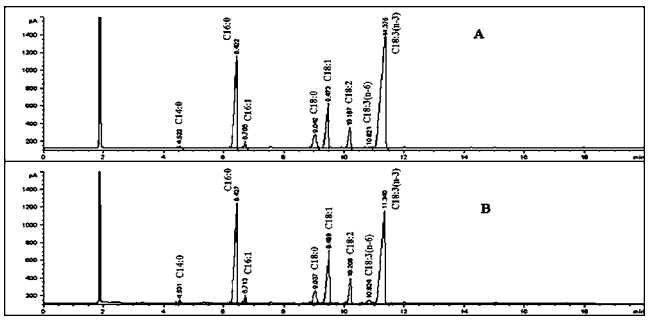

Figure 2. GC chromatogram of FAMEs of pupal oil extracted from eri silkworm grown on (**A**) tapioca and (**B**) castor leaves.

in pupal oil, followed by β-sitosterol. Saito et al. (*21*) also reported similar sterol compositions in silkworm of *B. mori* L. The influence of host plant was observed specifically in the level of cholesterol, showing significantly ($P < 0.001$) higher levels in the oil of pupae grown on castor leaves as compared to those grown on tapioca leaves. **Figure 1** shows the comparative GC chromatogram of sterols of oil extracted from silkworm grown on castor and tapioca leaves. The level of cholesterol in the pupal oil (approximately 60−90 mg/100 g of oil) is comparatively lower than other animal fats (85−110 mg/100 g of fat) (*22*).

The fatty acid composition of pupal oil extracted from eri silkworm grown on two different host plants was analyzed, and comparative GC profiles are given in **Figure 2**. ALA and palmitic acid are the two major fatty acids of pupal oil together comprising 72−78% followed by oleic, linoleic, and stearic acids (**Table 3**). The influence of host plant is pronounced in the fatty acid composition, particularly in the level of ALA, showing a significantly ($P < 0.001$) higher level in the oil of tapioca-based silkworm (58.3%) as compared to castor-based silkworm (42.9%). To understand such influence, the total lipid fatty acid composition of both castor and tapioca leaves were also analyzed and are included in **Table 3**. Tapioca leaves have a comparatively higher level of ALA (44.9%) than castor have (40.1%).

Table 3. Fatty Acid Composition (wt %) of Total Lipid of Castor and Tapioca Leaves and the Pupal Oil Extracted from Silkworm Grown on Castor and Tapioca Leaves[a]

	leaf total lipid FAME		pupal oil FAME	
fatty acid	castor	tapioca	castor	tapioca
14:0	1.1 ± 0.01	0.4 ± 0.02	0.5 ± 0.15	0.1 ± 0.10
16:0	29.4 ± 0.60	28.3 ± 0.05	29.2 ± 1.1	19.8 ± 0.53[b]
16:1	3.9 ± 0.52	3.0 ± 0.10	1.5 ± 0.11	0.8 ± 0.20
18:0	3.0 ± 0.28	4.1 ± 0.20	3.4 ± 0.15	4.5 ± 0.49
18:1	5.1 ± 0.05	4.2 ± 0.15	14.9 ± 0.42	10.2 ± 1.11[b]
18:2	16.8 ± 0.36	14.6 ± 0.1	5.4 ± 0.31	5.0 ± 0.21
18:3 (n-6)	0.2 ± 0.10	0.2 ± 0.10	0.2 ± 0.10	0.2 ± 0.11
18:3 (n-3)	40.1 ± 1.69	44.9 ± 2.17	42.9 ± 1.70	58.3 ± 2.1[b]

[a] Results are expressed as weight percentage of total fatty acids. Data are means ± SD for three samples. [b] Significantly different from castor group: $P < 0.001$.

Table 4. TAG Molecular Species Composition (%) of Oil Extracted from Silkworm Pupae Grown on Castor and Tapioca Leaves[a]

		pupae oil	
ECN	expected molecular species[b]	castor fed	tapioca fed
ECN36	LnLnLn	9.6 ± 0.53	20.2 ± 0.20[d]
ECN38	LLnLn	2.6 ± 0.51	4.3 ± 0.43
ECN40	LLLn/LnLnP/LnLnO	35.7 ± 0.42	42.4 ± 1.52[c]
ECN42	LLL/LLnO/PLLn/ SLnLn	6.9 ± 0.30	7.3 ± 0.61
ECN44	LnOO/OLL/SLLn/PLL/PLnP	28.8 ± 0.41	18.7 ± 0.41[d]
ECN46	POL/OOL/PLP	4.0 ± 0.30	2.3 ± 0.42
ECN48	SLnS/OOO/POP	11.5 ± 2.7	3.1 ± 0.21[d]

[a] Results are expressed as area percent of total TAG molecular species. Data are means ± SD for three samples. [b] Ln, α-linolenic acid; L, linoleic acid; O, oleic acid; P, palmitic acid; and S, stearic acid. [c] Significantly different from castor group: $P < 0.01$. [d] Significantly different from castor group: $P < 0.001$.

RP-HPLC analysis of TAG molecular species of oil showed some differences between the two groups, although the overall distribution profiles are identical (**Table 4** and **Figure 3**). The identification of molecular species is based on classification of TAGs containing five most abundant fatty acids as reported by Buchgraber et al. (*14*). The major molecular species in either oil is with ECN C40, and the content is significantly higher ($P < 0.01$) in the tapioca group than the castor group. The level of trilinolenin (LnLnLn) in oil with ECN C36 is significantly less ($P < 0.001$) in silkworm grown on castor leaves as compared to those grown on tapioca leaves. This is presumably due to a higher level of ALA in tapioca-based pupal oil.

The absorption behavior of individual fatty acids depends on their positional distribution over the glycerol backbone. A higher level of essential fatty acids (EFAs) at the *sn*-2 position of the TAG increases the bioavailability of such fatty acids, which are precursors for biosynthesis of their higher homologues such as arachidonic (AA), eicosapentanoic (EPA), and docosahexanoic acids (DHA) and their subsequent conversion to eicosanoids. In the present study, lipase-mediated regiospecific analysis of pupal oil was carried out and the results are given in **Table 5**. The results depict that the distribution of unsaturated fatty acids is more at the *sn*-2 position of the TAG backbone. The significantly higher concentration of ALA and lower concentration of palmitic acid at the *sn*-2 position ($P < 0.001$) in oil of silkworms grown on tapioca as compared to castor were

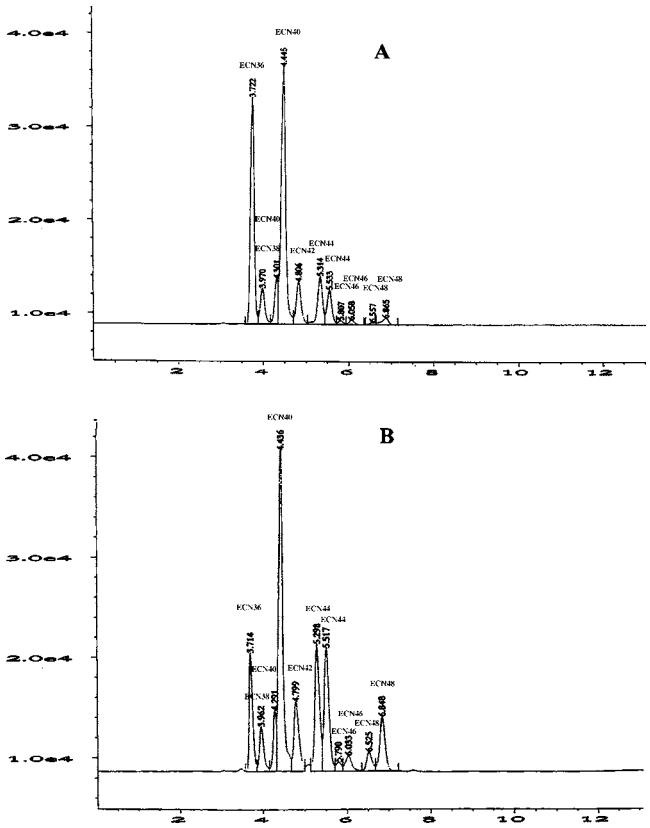

Figure 3. RP-HPLC chromatogram of TAG molecular species of pupal oil extracted from eri silkworm grown on (**A**) tapioca and (**B**) castor leaves. Peaks are assigned by ECNs. See **Table 4** for TAG molecular species corresponding to different ECNs.

Table 5. Fatty Acid Composition (%) at *sn*-2 and -1,3 Positions of TAGs in Oils Obtained from Silkworm Pupae Grown on Castor and Tapioca Leaves[a]

	pupal oil			
	castor fed		tapioca fed	
fatty acid	sn-2	sn-1,3	sn-2	sn-1,3
C14:0			0.8 ± 0.2	
C16:0	26.7 ± 2.8	43.7 ± 3.1	16.4 ± 4.1[b]	32.8 ± 3.5[b]
C18:0	1.6 ± 0.31	5.5 ± 0.81	1.3 ± 0.8	6.4 ± 1.1
C18:1	19.0 ± 2.5	13.5 ± 3.1	15.4 ± 1.5[b]	8.4 ± 2.2[b]
C18:2	5.2 ± 1.1	5.8 ± 2.1	4.9 ± 0.9	3.5 ± 0.6
C18:3 (n-6)	0.2 ± 0.1		0.9 ± 0.2	
C18:3 (n-3)	47.3 ± 3.1	31.5 ± 4.2	60.2 ± 5.5[b]	48.7 ± 3.8[b]

[a] Results are expressed as weight percentage of total fatty acids. Data are means ± SD for three samples. [b] Significantly different from castor group: $P < 0.001$.

observed, which reflect their presence in total oil. A higher level of ALA in the total oil and their predominance at the *sn*-2 position increase the nutritional importance of this oil.

ALA is a natural precursor of dietary long chain n-3 polyunsaturated fatty acids (PUFAs), like EPA and DHA. n-3 PUFAs are known to have positive effects on several risk factors associated with coronary heart disease (CHD) (*23*). An ALA-enriched diet could almost have similar favorable effects of EPA and DHA of fish oil in conferring protection against CHD (*24*). Sources of oil rich in linoleic acid are abundant in nature, but such is not the case with ALA. Thus, the oil extracted from desilked eri silkworm pupae offers an abundant and accessible source of ALA. The observed differences in the level of ALA in oils extracted from silkworm pupae grown on two different host plants may be attributed to the type of host plants. The influence of host plant is also observed in the level of cholesterol in the extracted pupal oil. The level of cholesterol in the pupal oil is comparatively lower than other animal fats (*22*). Thus, the beneficial effect of the studied oil in conferring protection against CHD due to the presence of a large amount of ALA may reduce the risk factors associated with a relatively higher content of cholesterol.

ABBREVIATIONS USED

ALA, α-linolenic acid; TAG, triacylglycerol; EPA, eicosapentaenoic acid; DHA, docosahexanoic acid; PUFAs, polyunsaturated fatty acids; FAME, fatty acid methyl ester; RP-HPLC, reversed-phase high-performance liquid chromatography; ECN, equivalent carbon number; EFAs, essential fatty acids.

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