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Effects of Dietary Anthocyanins on Tocopherols and Lipids in Rats

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The effects of dietary cyanidin-3-*O*-glucoside (C3G) and concentrates from blackcurrant [*Ribes nigrum*] (BC) and elderberry [*Sambucus nigra*] (EC) on plasma and tissue concentrations of α- (α-T) and γ-tocopherol (γ-T) and cholesterol, as well as the fatty acid composition of the liver lipids were investigated in growing, male rats of the Sprague−Dawley strain. Animals were fed semisynthetic diets supplemented with 2 g/kg C3G, BC, or EC for 4 weeks. Dietary anthocyanins did not affect feed intake, body weight, and organ weights. C3G elevated the concentrations of tocopherols in the liver and lungs ($P < 0.05$). Cholesterol levels in plasma and liver were not affected by any of the regimens. C3G and BC reduced the relative amount of saturated fatty acids in the liver ($P < 0.05$). BC also lowered the percentage of 22:6 + 24:0 and EC the ratio of 20:3/20:4 n-6 ($P < 0.05$). In conclusion, dietary C3G, BC, and EC appear to have little effect on cholesterol levels and the fatty acid pattern in the liver but seem to be capable of sparing vitamin E in healthy, growing rats.

KEYWORDS: Anthocyanins; cholesterol; cyanidin-3-*O*-glucoside; fatty acids; rats; tocopherols

INTRODUCTION

Scientific interest in compounds from the plant secondary metabolism, the so-called phytochemicals, is strong, because epidemiological studies suggest that diets with a high intake of vegetables and fruits may reduce the incidence of degenerative diseases (*1*). Anthocyanins represent the predominant class of water-soluble pigments in plants responsible for most of the blue, red, and intermediate colors. Their chemical structure, consisting of an anthocyanidin (flavonoid) skeleton with mainly 3-*O*-glycosidic or 3,5-di-*O*-glycosidic bound sugar residues, gives rise to several hundred different forms. The major glycosidic sugars in plant tissues are glucose, galactose, rhamnose, and arabinose. The aglycones are rarely found in plants, but the most widespread anthocyanidin in the plant kingdom is cyanidin (*2*, *3*). Anthocyanins occur frequently in food items such as berries, leafy vegetables, cereals, tubers, bulbs, juices from berries, and red wines and, thus, play an important role in human and animal nutrition (*3*, *4*). In 1976, Kühnau (*4*) estimated the average daily intake of anthocyanins in the U.S. American population to be 180−215 mg. Because red wine contains considerably high amounts of anthocyanins, regular consumers are likely to have even higher daily intakes (*5*).

Direct intestinal absorption of orally administered anthocyanins was demonstrated in rats and humans (*6*, *7*). Youdim et al. (*8*) observed the incorporation of anthocyanins into the membrane and cytosol of vascular endothelial cells and an increased protection against oxidative insult. In rats, anthocyanins were reported to increase the ex vivo oxidation resistance of blood serum (*9*) and to counteract lipid peroxidation and DNA damage induced by vitamin E-depletion in hepatocytes (*10*). In human colon cells, on the other hand, pure anthocyanins and mixtures thereof did not reduce oxidative DNA damage but served as powerful antioxidants in vitro (*11*). In line with these observations, other investigators demonstrated that anthocyanins exert antioxidant and antiinflammatory activity in vitro (*12*−*15*). Anthocyanins were also shown to have modulating effects on enzyme systems, such as cyclooxygenase (*16*) and prostaglandin endoperoxide synthase (*15*).

A growing body of evidence suggests the involvement of an excess formation of free radicals in the development of various pathological conditions, such as atherosclerosis, cardiovascular disease, stroke, cancer, arthritis, and Alzheimer's disease (*17*). Dietary antioxidants such as anthocyanins may be helpful in the prevention of those conditions by counteracting an imbalance of oxidative and antioxidative factors in the living organism.

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Table 1. Composition of the Basal Diet[a]

ingredient	g/kg
maize starch	528
casein (vitamin-free)	200
rapeseed oil[b]	100
sucrose	80
cellulose powder	40
mineral and trace element premix[c]	40
vitamin premix (vitamin E-free)[c]	10
cholesterol[d]	2

[a] Anthocyanin concentrates were added to the experimental diets at a concentration of 2 g/kg. [b] All vitamin E in the diet originated from the rapeseed oil. Tocopherol and tocotrienol concentrations were determined according to IUPAC 2.432 and were as follows: α-tocopherol 291 ppm, γ-tocopherol 355 ppm, δ-tocopherol <10 ppm; α-, γ- and δ-tocotrienols were present at concentrations less than 10 ppm. The fatty acid composition of the rapeseed oil, determined according to IUPAC 2.302, was 16:0 (4.3%), 16:1 (0.2%), 17:1 (0.2%), 18:0 (1.5%), 18:1 (62.5%), 18:2 (19.6%), 18:3 (9.5%), 20:0 (0.5%), 20:1 (1.3%), 22:0 (0.3%), and 22:1 (0.3%). [c] The mineral and trace element premix and the vitamin premix were formulated to meet the nutritional requirements of laboratory rats with the exception of vitamin E and purchased from Lactamin (Lidköping, Sweden). [d] The cholesterol was dissolved in ethanol and sprayed onto the diet.

At present, information on the interactions of anthocyanins with the antioxidant defense system in the body is scarce.

In the course of our efforts to screen a range of dietary phenolic compounds for their potential effects on vitamin E and lipid metabolism, we tested the effects of orally administered cyanidin-3-*O*-glucoside and two anthocyanin concentrates from blackcurrant [*Ribes nigrum*] and elderberry [*Sambucus nigra*] fruits on tocopherol and lipid levels in a rat model.

MATERIALS AND METHODS

Experimental Animals and Diets. We used 28 male, 21 d old Sprague−Dawley rats with a mean body weight of 62 g (B&K Universal AB, Sollentuna, Sweden) for this study. The animals were housed individually in Macrolon IV cages (Ehret GmbH & Co., Emmendingen, Germany) with aspen wood bedding (Beekay bedding, B&K Universal AB) in a conditioned room at 25 °C and 60% relative humidity with 12 h light (07:00 to 19:00) and 12 h darkness. Each cage was equipped with a water bottle with metal lid, a feed container attached to a stainless steel plate to avoid overthrowing and spilling, two black plastic tubes which the rats used for resting and hiding, and a table tennis ball for playing. The rats had free access to feed and water throughout the experiment, which was carried out in accordance with the guidelines of and approved by the Ethical Committee for Animal Experiments in the Uppsala region. The composition of the basal diet is shown in **Table 1**. Rapeseed oil was a gift from Karlshamns AB (Karlshamn, Sweden). Cholesterol (C), purchased from Sigma Chemical Co. (St. Louis, MO), cyanidin-3-*O*-glucoside (C3G; Polyphenols AS, Sandnes, Norway), blackcurrant (BC), and elderberry concentrates (EC; Dr. Marcus GmbH, Geesthacht, Germany) were added to the basal diet at concentrations of 2 g/kg. According to the manufacturer, the berry concentrates do not contain detectable amounts of ascorbic acid.

Analysis of Anthocyanins, Flavonol Glycosides, and Hydroxycinnamic Acids. Adequate samples of C3G, freeze-dried BC, and EC were dissolved in small aliquots of 6 M hydrochloric acid (HCl) in order to release anthocyanins. Methanol was added until HCl was diluted to 0.6 M. Dissolved samples were mixed thoroughly and filtered through a 0.45 μm Regen Cellulose syringe filter (TITAN, Gloucester, UK) prior to analysis. Identification and quantification of anthocyanins, flavonol glycosides, and hydroxycinnamic acids were performed as described earlier (*18*). The standard curves of anthocyanins as delphinidin- and cyanidin-3-*O*-β-glucoside ranged from 6 to 1800 μg of aglycon in 1 mL of 0.6 M HCl in methanol and of flavonol glycosides as quercetin-3-*O*-rutinoside ranged from 2 to 300 μg of quercetin aglycon in 1 mL of methanol. The phenolic composition of C3G, BC, and EC is shown in **Table 2**. The C3G powder contained 331.6 mg/g cyanidin-3-*O*-glucoside and 115.5 mg/g cyanidin aglycon. The BC contained 18.2 mg/g of a mixture of cyanidin- and delphinidin-glucosides and -rutinosides. The EC consisted of 116.7 mg/g of a mixture of three cyanidin-glycosides of which two major ones eluted as one unseparated peak. According to literature, cyanidin-3-sambubioside and cyanidin-3-glucoside are found as the major anthocyanins in elderberry (*19*).

Table 2. Detected Anthocyanins in the Cyanidin-3-*O*-glucoside,[a] Blackcurrant,[b] and Elderberry[c] Concentrate[d]

compound	conc. (mg/g)
Cyanidin-3-*O*-glucoside Concentrate	
cyanidin-3-*O*-glucoside	331.6
cyanidin (aglycon)	115.5
total	447.1
Blackcurrant Concentrate	
cyanidin-3-*O*-glucoside	0.9
cyanidin-3-*O*-rutinoside	6.7
delphinidin-3-*O*-glucoside	2.9
delphinidin-3-*O*-rutinoside	7.8
total	18.2
Elderberry Concentrate	
cyanidin-3–5-diglucoside	10.8
cyanidin-3-*O*-glucoside + cyanidin-3-*O*-sambubiose	105.9
total	116.7

[a] No flavonols were detected in the C3G concentrate. [b] The blackcurrant concentrate contained 0.92 mg/g flavonols. [c] The elderberry concentrate contained 6.9 mg/g flavonols. [d] No hydroxycinnamic acid derivatives were detected in the concentrates.

Study Design and Analyses. The 28 rats were divided into 4 groups of 7 animals with similar mean body weights and fed their respective diets ad libitum for 4 weeks. Body weights were measured weekly. At the end of the experiment, the rats were fasted for 12 h before intraperitoneal injection of an overdose of sodium pentobarbital and killed by exsanguination. Blood samples were withdrawn from the *vena cava*, collected in EDTA tubes, and centrifuged (1000 × g, 10 min), and the blood plasma was transferred to test tubes with screw caps and stored at −20 °C until analyzed. Liver and lung tissues were excised, weighed, transferred into 2-propanol-filled test tubes, and stored at −80 °C. Plasma and tissue lipids were extracted and analyzed for tocopherols and cholesterol as described before (*20*). Liver lipids were also analyzed for fatty acid composition. In blood plasma, the lipoprotein fractions were separated and the tocopherol and cholesterol levels determined (see *20*). Triacylglycerols (TAG) were quantified in the plasma and in the isolated lipoprotein fractions according to the IL test triglyceride enzymatic-colorimetric method 181610−60 employing a Monarch apparatus (Instrumentation Laboratories, Lexington, MA).

Statistical Analyses. Statistical analysis of the registered variables was performed by an analysis of variance procedure and the general linear model supported by the Statistical Analysis System (*21*). Least significant differences from the *t*-test function of the SAS general linear model procedure were used to make statistical comparisons and effects were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

In our effort to screen dietary phytochemicals for potential physiological effects, we chose the pure anthocyanin cyanidin-3-*O*-glucoside (C3G) and anthocyanin mixtures in the form of concentrates from blackcurrant (BC) and elderberry (EC), because of their abundance in the human and animal diet. Orally applied anthocyanins are absorbed intact in their glycated form and can be detected in plasma, liver, and urine of humans and rats (*6*, *7*, *18*, *22*−*24*). As mentioned before, the daily intake of anthocyanins in the USA has been estimated to be 215 mg during summer and 180 mg during winter disregarding potentially higher intakes in regular red wine drinkers (*4*, *5*). For a

Table 3. Body and Organ Weights[a]

diet:	control $n = 7$	cyanidin-3-*O*-glucoside $n = 7$	black currant concentrate $n = 7$	elderberry concentrate $n = 7$	$P <$
body weight (g)	230.8 ± 7.5	227.3 ± 12.8	232.8 ± 9.9	231.5 ± 8.1	n.s.
liver weight (g)	10.3 ± 0.5	10.4 ± 0.9	10.9 ± 1.7	10.2 ± 0.8	n.s.
relative liver weight (g/100 g BW)[b]	4.4 ± 0.3	4.6 ± 0.6	4.8 ± 0.7	4.4 ± 0.4	n.s.
lung weight (g)	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.1 ± 0.0	n.s.
relative lung weight (g/100 g BW)	0.5 ± 0.0	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	n.s.

[a] n = number of observations, values represent means ± SEM; n.s. = no statistical significance. [b] BW = body weight.

Table 4. Effect of Anthocyanins on Tocopherol Concentrations (μg/dL) in Rat Plasma and Liver (μg/g)[a]

diet:	control	cyanidin-3-*O*-glucoside	black currant concentrate	elderberry concentrate	$P <$
			Plasma (mg/dL)		
	$n = 7$	$n = 7$	$n = 7$	$n = 7$	
α-T	2.42 ± 0.17	2.56 ± 0.26	2.40 ± 0.36	2.77 ± 0.64	n.s.
γ-T	0.16 ± 0.08	0.21 ± 0.12	0.16 ± 0.07	0.26 ± 0.14	n.s.
α-T + γ-T	2.59± 0.24	2.77 ± 0.36	2.56 ± 0.42	3.03 ± 0.76	n.s.
γ-T/α-T	0.07 ± 0.03	0.08 ± 0.04	0.07 ± 0.03	0.09± 0.03	n.s.
			Liver (mg/g)		
	$n = 7$	$n = 7$	$n = 7$	$n = 7$	
α-T	7.56 ± 0.78[b]	8.84 ± 0.67[c]	7.27 ± 0.79[b]	7.64 ± 0.94[b]	0.05
γ-T	1.24 ± 0.22[b,c]	1.54 ± 0.30[b]	1.20 ± 0.34[c]	1.37 ± 0.26[b,c]	0.05
α-T + γ-T	8.80 ± 0.61[b]	10.39 ± 0.82[c]	8.47 ± 0.76[b]	9.01 ± 1.15[b]	0.05
γ-T/α-T	0.17 ± 0.05	0.17 ± 0.03	0.17 ± 0.05	0.18 ± 0.02	n.s.
			Lung (mg/g)		
	$n = 7$	$n = 6$			
α-T	10.23 ± 1.05	11.35 ± 0.88	n.a.	n.a.	n.s.
γ-T	1.49 ± 0.12[b]	1.90 ± 0.36[c]	n.a.	n.a.	0.01
α-T + γ-T	11.71 ± 1.00[b]	13.25 ± 1.17[c]	n.a.	n.a.	0.05
γ-T/α-T	0.15 ± 0.02	0.17 ± 0.02	n.a.	n.a.	n.s.

[a] n = number of observations, values represent means ± SEM; n.a. = not analyzed; n.s. = no statistical significance. [b,c] Values within each row not sharing a common superscript letter are statistically different at P.

human of 70 kg body weight, this corresponds to a daily consumption of 3.1 and 2.6 mg anthocyanins per kg body weight, respectively. During this experiment, the rats eating the C3G-supplemented diet consumed 4.6−8.6 mg/kg/d, the animals on the BC diet ate 0.3−0.5 mg/kg/d, and those on the EC diet ate 1.6−3.0 mg/kg/d of the concentrates. Thus, the dietary intake of anthocyanins during this study is of the same order of magnitude as that expected in humans.

Dietary supplementation with C3G, BC, and EC for four weeks did not affect animal performance. Feed intake, body weight, and the total and relative (g/100 g body weight) weights of liver and lungs were similar in all rats (**Table 3**). These findings are in accordance with previously published data (*9*, *10*) and the low toxicity of anthocyanins (*3*).

The rats in the present experiment were deprived of their respective diets at least 12 h before tissue samples were collected. It is known that C3G is rapidly absorbed and metabolized in rats. Approximately 4 h after the ingestion of a single dose of C3G (0.9 mmol/kg body weight), concentrations of C3G, cyanidin, and protocatechuic acid, a metabolite of C3G, in blood plasma, stomach, jejunum, liver, and kidneys, dropped to baseline levels again (*6*, *23*). Thus, the fasting period in this experiment seems to be sufficient to eliminate the ingested anthocyanins from the body, which should be kept in mind for the interpretation of the results.

In blood plasma, no significant changes in α- (α-T) and γ-tocopherol (γ-T) levels (**Table 4**) were observed in any of the experimental groups, although C3G and the EC showed a tendency toward elevating both tocopherols. In the liver, only C3G elevated α-T and the sum of α-T and γ-T statistically ($P < 0.05$) and γ-T numerically. Because experience from earlier studies suggested that effects on tocopherol concentrations may be more pronounced in the lungs, we analyzed those from the C3G-supplemented rats and found γ-T ($P < 0.01$) and the sum of the two tocopherols ($P < 0.05$) still to be significantly but α-T to be only numerically elevated. In previous experiments, C3G feeding (2 g/kg diet for 14 d) did neither affect serum levels of α-T in 5-week-old male rats (*9*) nor serum and liver α-T concentrations in a rat model for liver ischemia-reperfusion injury (*25*). Similarly, feeding vitamin E-depleted rats a diet fortified with 1 g/kg anthocyanins for two weeks did not change α-T concentrations in plasma and liver (*10*). These differences may be due to the shorter feeding periods in these studies (2 weeks instead of 4 weeks in our experimental design) and an increased requirement of antioxidants in the liver ischemia-reperfusion injury model and the vitamin E-depleted rats.

None of the three regimens affected the concentrations of total cholesterol, cholesterol in the lipoprotein fractions, and triacylglycerols in plasma nor total cholesterol, the amount of lipids, and the percentage of cholesterol in the liver (**Table 5**). Tsuda et al. (*9*), on the other hand, reported reduced concentrations of total and free cholesterol in serum from C3G-supplemented rats, whereas concentrations of triacylglycerols, phospholipids, free fatty acids, and esterified cholesterol were not changed.

With respect to the fatty acid pattern of the liver lipids, dietary supplementation with C3G and BC lowered the amount of total saturated fatty acids (SFA; $P < 0.05$) as displayed in **Table 6**. All treatments showed a tendency toward reducing the percentage of 22:6 + 24:0, with the effect being significant in the BC-group only ($P < 0.05$). The ratio of 20:3/20:4 n-6, a marker of

Table 5. Effect of Anthocyanins on Cholesterol and Triacylglycerols in Blood Plasma and Cholesterol and Total Lipids in the Liver[a]

diet:	control	cynidin-3-*O*-glucoside	black currant concentrate	elderberry concentrate	*P* <
		Plasma (mg/dL)			
	n = 7	*n* = 7	*n* = 6	*n* = 7	
total cholesterol	83.96 ± 10.23	77.33 ± 9.52	79.13 ± 10.67	90.86 ± 19.82	n.s.
HDL	28.44 ± 2.87	29.22 ± 5.09	27.58 ± 5.45	29.38 ± 5.03	n.s.
VLDL + LDL	55.51 ± 10.05	48.11 ± 12.30	51.55 ± 11.13	61.48 ± 16.43	n.s.
HDL/TC	0.34 ± 0.05	0.39 ± 0.11	0.35 ± 0.07	0.33 ± 0.06	n.s.
triacylglycerols	101.15 ± 47.62	109.87 ± 40.54	108.42 ± 35.74	98.87 ± 39.09	n.s.
		Liver (mg/g liver)			
	n = 7	*n* = 7	*n* = 7	*n* = 6	
total cholesterol	19.79 ± 4.36	22.78 ± 1.56	22.03 ± 4.05	21.03 ± 4.02	n.s.
liver lipids	91.14 ± 15.85	102.00 ± 15.29	96.29 ± 12.37	104.17 ± 16.68	n.s.
% TC in liver lipids	21.84 ± 3.51	22.79 ± 3.52	22.86 ± 2.56	22.50 ± 5.12	n.s.

[a] *n* = number of observations, values represent means ± SEM; n.s. = no statistical significance.

Table 6. Effect of Anthocyanins on the Fatty Acid Composition of Liver Lipids[a]

diet:	control	cynidin-3-*O*-glucoside	black currant concentrate	elderberry concentrate	*P* <
relative fatty acids (%)	*n* = 7	*n* = 7	*n* = 7	*n* = 7	
14:0	0.77 ± 0.11	0.80 ± 0.10	0.76 ± 0.08	0.83 ± 0.08	n.s.
16:0	20.43 ± 1.60	18.89 ± 1.97	19.52 ± 1.25	20.55 ± 1.97	n.s.
18:0	6.08 ± 1.10	5.67 ± 1.26	5.21 ± 0.74	5.16 ± 0.81	n.s.
SFA[b]	27.29 ± 1.51[c]	25.36 ± 2.29[d]	25.49 ± 1.04[d]	26.54 ± 1.38[c,d]	0.05
16:1	4.93 ± 0.83	4.29 ± 0.86	4.70 ± 0.95	5.50 ± 1.44	n.s.
18:1	42.72 ± 2.02	43.06 ± 2.55	44.83 ± 1.83	44.12 ± 2.84	n.s.
MUFA[b]	47.64 ± 2.37	47.35 ± 3.20	49.54 ± 2.68	49.63 ± 3.84	n.s.
18:2	12.14 ± 1.42	13.84 ± 2.02	12.79 ± 1.66	11.79 ± 2.46	n.s.
18:3 n-6	0.16 ± 0.03	0.19 ± 0.07	0.19 ± 0.05	0.18 ± 0.04	n.s.
20:3	0.47 ± 0.10	0.44 ± 0.09	0.37 ± 0.07	0.37 ± 0.08	n.s.
20:4	5.40 ± 1.16	5.25 ± 1.40	4.78 ± 0.71	4.83 ± 0.88	n.s.
n-6	18.17 ± 2.09	19.71 ± 2.46	18.13 ± 2.29	17.17 ± 3.31	n.s.
18:3 n-3	2.14 ± 0.46	2.59 ± 0.59	2.41 ± 0.45	2.22 ± 0.59	n.s.
20:5	1.09 ± 0.20	1.14 ± 0.21	0.99 ± 0.12	1.03 ± 0.24	n.s.
22:5	0.81 ± 0.22	0.90 ± 0.26	0.79 ± 0.16	0.76 ± 0.41	n.s.
22:6+24:0	3.17 ± 0.64[c]	2.93 ± 1.06[c,d]	2.40 ± 0.23[d]	2.61 ± 0.26[c,d]	0.05
n-3	7.22 ± 1.00	7.55 ± 1.30	6.58 ± 0.78	6.63 ± 1.29	n.s.
PUFA[b]	25.39 ± 2.93	27.26 ± 3.47	24.72 ± 2.75	23.80 ± 4.58	n.s.
n-6/n-3	2.54 ± 0.23	2.64 ± 0.28	2.77 ± 0.37	2.59 ± 0.12	n.s.
20:3/20:4	0.09 ± 0.00[c]	0.08 ± 0.01[c,d]	0.08 ± 0.01[c,d]	0.08 ± 0.01[d]	0.05
18:3 n-3/20:5	2.03 ± 0.59	2.34 ± 0.63	2.43 ± 0.25	2.16 ± 0.37	n.s.

[a] *n* = number of observations, values represent means ± SEM. [b] SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. [c,d] Values within each row not sharing a common superscript letter are statistically different at P; n.s. = no statistical significance.

Δ^5-desaturase activity, was statistically reduced by EC ($P < 0.05$) and numerically by C3G and BC.

The results of the present investigation suggest that cyanidin-3-*O*-glucoside and anthocyanin mixtures from blackcurrant and elderberry consumed at normal dietary levels do only have little effects on cholesterol values and the liver fatty acid pattern in healthy, well-nourished growing rats. Previously, C3G and anthocyanin concentrates have been reported to suppress oxidative damage in vivo (*6*, *9*, *10*, *26*). A direct effect of anthocyanins on tocopherol concentrations in vivo, on the other hand, has not been shown before. As suggested by our results, owing to their redox properties, anthocyanins may have the potential to work synergistically with vitamin C and the antioxidant defense system in sparing vitamin E. Further studies on the effects of anthocyanin-rich berry extracts on tocopherol levels in humans are underway.

ABBREVIATIONS USED

BC, blackcurrant concentrate; C3G, cyanidin-3-*O*-glucoside; EC, elderberry concentrate; HCl, hydrochloric acid; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; T, tocopherol; TAG, triacylglycerol; VLDL, very low-density lipoprotein.

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