Trans n-3 eicosapentaenoic and docosahexaenoic acid isomers exhibit different inhibitory effects on arachidonic acid metabolism in human platelets compared to the respective *cis* fatty acids

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Abstract N-3 *trans* geometrical isomers of 20:5 n-3 and 22:6 n-3 were isolated from rats fed heated linseed oil. The ability of these acids to inhibit 20:4 n-6 metabolism by human platelets was examined. The concentrations required to inhibit 50% of platelet aggregation (IC50) induced by 2.5 μM 20:4 n-6 were higher for the 20:5 Δ17*t* isomer compared to all *cis* 20:5 n-3; means 29.2 and 7.6 μM, respectively ($P < 0.05$). There were no significant differences in IC50 between 22:6 Δ19*t* and all *cis* 22:6 n-3; means 4.3 and 5.6 μM, respectively ($P > 0.05$). Inhibition of action of cylooxygenase on 20:4 n-6 was similar for 20:5 Δ17*t* and 20:5 n-3 when examined at their IC50s, but comparison at equal concentrations indicated that 20:5 n-3 was a significantly better inhibitor ($P < 0.05$). The ability to inhibit platelet aggregation was paralleled by cyclooxygenase inhibition as determined by thromboxane B_2 and 12-hydroxyheptadecatrienoic acid formation. 22:6 Δ19*t* appeared to inhibit cyclooxygenase more completely than 22:6 n-3, examined at their IC50s or at similar concentrations ($P < 0.05$). Isomers of 20:5 n-3 and 22:6 n-3 having an n-3 *cis* or *trans* bond appear to have similar modes of action, although levels required for effectiveness are different for the C20 acids. —**O'Keefe, S. F., M. Lagarde, A. Grandgirard, and J. L. Sebedio.** *Trans* n-3 eicosapentaenoic and docosahexaenoic acid isomers exhibit different inhibitory effects on arachidonic acid metabolism in human platelets compared to the res- pective *cis* fatty acids. *J. Lipid Res.* 1990. **31:** 1241-1246.

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Geometrical isomers of unsaturated fatty acids are formed in oils during heat treatment, including deodorization (1) and deep-fat frying (2-4). Mono and di-*trans* isomers of linoleic and linolenic acids have been identified in oils taken from commercial users in France (5). A geometrical isomer of all *cis*-5, 8, 11, 14, 17- eicosapentaenoic acid (EPA) containing a *trans*-17 bond (20:5 Δ17*t*) has been identified in the liver lipids of rats fed heated linseed oil (6). Recently, an isomer of all *cis*- 4, 7, 10, 13, 16, 19-docosahexaenoic acid (DHA) with a *trans*-19 bond (22:6 Δ19*t*) was identified in similarly fed rats (7). These *trans* fatty acid isomers could be formed by elongation and desaturation of an 18:3 n-3 geometrical isomer, 18:3 Δ9*c*, 12*c*, 15*t*, present in the heated oil. An isomer of linoleic acid, 18:2 Δ9*c*, 12*t*, can be metabolized into a mono-*trans* isomer of arachidonic acid (AA) (8, 9).

Although it is clear that geometrical isomers of physiologically important C20 and C22 fatty acids may be formed in vivo, little is known of the metabolic fate of these unusual fatty acids. The structural difference between AA and EPA is only the presence of an n-3 *cis* bond in the latter, yet the roles of these fatty acids are distinctly different. The presence of 20:5 Δ17*t* in vivo is disturbing because of its structural similarity to AA. In this study, the effect of n-3 double bond geometry in EPA and DHA on inhibition of AA metabolism in isolated human platelets has been examined.

MATERIALS AND METHODS

Chromatography

High performance liquid chromatography was carried out on preparative (Prep-500A; Prep-Pak C-18 column; Waters, MA; acetonitrile 200 ml/min), semi-preparative

Abbreviations: AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FAME, fatty acid methyl esters; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; FFA, free fatty acids; HHT, 12-hydroxyheptadecatrienoic acid; HETE, 12-hydroxyeicosatetraenoic acid; TXB_2, thromboxane B_2.

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(Model 6000A pump, 510 refractive index detector, Valco injector; Waters; Hybar 7 μ C-18 column; Merck, FRG; methanol 3.0 ml/min), and analytical (0.8 × 10 cm C-18 column; Waters; methanol–water 9:1 (v/v), 1.2 ml/min) scales.

Gas–liquid chromatography was carried out on Packard or Girdel units fitted with 0.32 mm × 30 m fused silica columns (DB-Wax, J&W Scientific or CP Sil 88, Chrompak). Injections were done by using Ros solid injectors. All solvents were redistilled in glass before use.

Isolation of n–3 fatty acids

EPA, DHA, and their n–3 *trans* isomers were isolated from the liver lipids of rats fed heated linseed oil. Thirty-week-old rats were obtained from a commercial source. For 4 weeks they were fed a purified equilibrated diet containing 10% (by wet weight) linseed oil that had been heated under nitrogen for 12 h at 275°C (10). At the end of 4 weeks, the rats were killed by decapitation and their livers were removed and stored in chloroform– methanol 2:1 (v/v) at –20°C until use (up to 6 weeks). The liver lipids were extracted by the method of Folch, Lees, and Sloane Stanley (11). Fatty acid methyl esters (FAME) were prepared by the method of Morrison and Smith (12).

The FAME were fractionated by using the chromatographic procedures described by Grandgirard et al. (7). Briefly, preparative and semi-preparative HPLC using C-18 columns was followed by silver nitrate thin-layer chromatography ($AgNO_3$-TLC) (13). Bands on the TLC plates were visualized by using dichlorofluorescein and UV light and were extracted by using the method of Hill, Husbands, and Lands (14). At this point, DHA, EPA and 22:6 $\Delta 19t$ were sufficiently pure (>99.5%) as determined by gas–liquid chromatography (GLC). However, 20:5 $\Delta 17t$ was contaminated with two components that together totalled about 10% of the GLC chromatogram area. These compounds were removed by using an analytical C-18 HPLC column eluted with methanol–water 9:1 (v/v) at 1.2 ml/min. Under these conditions, one peak eluted around 10.3 min and the peak was collected in two halves: the first half contained 99.3% 20:5 $\Delta 17t$, and the second 92.5% 20:5 $\Delta 17t$. The first half of the peak was collected several times to provide enough material for the experiments.

About 50 mg of the purified FAME was saponified by using 5 ml KOH (0.255 M, aq) with heating at 80°C for 65 min under a reflux condensor swept with nitrogen, as suggested by Lamothe et al. (15) for cod liver oil. The hydrolysis was complete under these conditions as determined by using the TLC-FID Iatroscan method (16). The extracted free fatty acids (FFA) were checked for artifact formation by transmethylation and GLC. The fatty acids were found to retain their purity after hydrolysis. The fatty acids were quantified at the FAME stage by using 21:0 internal standard and theoretical response factors (17). The FFA were stored in dilute ethanol solution under nitrogen at –80°C until use (up to 8 months). The purity was routinely assessed by transmethylation of an aliquot and analysis by GLC.

Platelet isolation and aggregation studies

Blood samples from healthy volunteers who had not taken any drugs for at least 10 days were provided by a local Blood Bank. Platelets were prepared from freshly collected human blood by using the method of Lagarde et al. (18). Briefly, platelet-rich plasma was acidified to pH 6.4 with citric acid and centrifuged for 10 min at 900 *g*. The platelet pellets were resuspended in Tyrode-HEPES pH 7.35 buffer and the platelet number was adjusted to 2.5×10^8/ml before use.

Aggregation studies were performed with the turbidimetric method of Born (19). Platelet suspensions (400 μl) were placed into glass turbidity tubes and warmed at 37°C with no stirring for 2 min in a Labintec aggregometer. A small magnetic stirring bar was then added along with 2 μl of the test fatty acid solutions made up in ethanol (95%); thus, the final ethanol concentration was 0.5%. Separate syringes were used to add 1μl of AA and either test n–3 fatty acid or 1 μl ethanol for the blank. The aggregation was followed for 4 min with a strip chart recorder. The 100% and 0% transmissions were adjusted by using buffer and platelet suspension, respectively.

The AA concentration was held constant at 2.5 μM for all experiments and a range of n–3 fatty acid concentrations was examined (1–80 μM). Levels of n–3 fatty acids were adjusted to produce inhibition from about 10 to 90% of the aggregation due to AA alone. Three to five data points were collected at least in duplicate for each fatty acid combination.

The concentration of n–3 fatty acid that produced 50% inhibition of aggregation (IC50) was determined by linear regression of the quasi-linear area between 20 and 80% aggregation. Small concentration differences of n–3 fatty acid around the IC50 produced large changes in inhibition. The IC50 conveniently describes the center of the aggregation-concentration curve. A Wilcoxon signed rank nonparametric test was used (20) to compare IC50s with a 5% level of significance.

The C20 fatty acids were evaluated on platelets from different donors than those for the C22 acids. This was necessary because the platelets retain their activity in vitro for a limited time. Platelets from 17 different donors were used, 9 for C20 and 8 for C22 fatty acids.

Thin-layer chromatography of [^{14}C]AA and its metabolites

[^{14}C]AA was obtained from CEA (Gif-sur-Yvette, France). Specific activity was 58 mCi/mmol. Thus, 1 nmol [^{14}C]AA (58 nCi) was used per 10^8 platelets. Radioactivity was detected by using a Berthold automatic TLC linear analyser (model LB 2832).

After 4.0 min of platelet incubation with fatty acids, the aggregation was stopped by addition of 1.2 ml of ethanol. The lipids were extracted with chloroform–methanol, separated by TLC, and metabolites were identified as described elsewhere (21, 22). Briefly, a two-step development scheme was used. A 90-min first development in hexane–diethyl ether–acetic acid 60:40:1 (v/v) separated free acid, 12-hydroxyheptadecatrienoic acid (HHT), and 12-hydroxyeicosatetraenoic acid (HETE) while polar lipid and TXB_2 remained at the origin. The polar lipid and TXB_2 were separated by using a 90-min second development with diethyl ether–methanol–acetic acid 90:1:2 (v/v). For some experiments the following modification was used. The more polar solvent was used for 12 min, the plate was dried briefly, and the former less polar solvent was used for 90 min. This modification did not significantly affect metabolite concentrations ($P > 0.05$, n = 8).

To compare cyclooxygenase inhibition by the n-3 fatty acids, the following calculation was performed. The concentration of the metabolites at 50% inhibition of aggregation was calculated by the linear regression of percent inhibition versus metabolite concentration over the range of aggregation from 20 to 80%.

RESULTS AND DISCUSSION

The isolation procedure was effective in obtaining highly pure fatty acids (>99.3%) as determined by GLC. The purity, total mass isolated, fold-purification, and % recovery from this procedure are shown for 20:5 Δ17*t* in **Table 1**. There was no noticeable degradation of these fatty acids during storage at −80°C in dilute solution in sealed tubes under nitrogen during the 8 months after isolation. Storage at −20°C or storage under atmosphere gas allowed noticeable oxidation after several months.

None of the four n-3 fatty acids examined, EPA, 20:5 Δ17*t*, DHA, and 22:6 Δ19*t*, induced platelet aggregation when added alone at the concentrations examined (1–30 μM). AA (2.5 μM) induced aggregation from 40 to 90% in the different blood samples. Both EPA and 20:5 Δ17*t* inhibited AA-induced aggregation. **Fig. 1** shows a typical aggregation pattern and the inhibition by 20:5 Δ17*t*. The width of the aggregation tracing often varied within one fatty acid combination so the percentage aggregation was determined from the center of the tracing. The pattern of inhibition in one platelet sample is illustrated in **Fig. 2** and the IC50s are shown in **Table 2**. The mean IC50 was significantly lower ($P < 0.05$) for EPA compared to 20:5 Δ17*t*, 7.6 and 29.2 μM, respectively. The marked variation between platelet preparations necessitated a paired statistical test such as a paired *t*-test, two-way analysis of variance (parametric tests), or the Wilcoxon signed rank (nonparametric). The nonparametric test was chosen because the assumption of normality in IC50s of the sampled population was not necessary.

EPA significantly decreased production of the cylooxygenase metabolites TXB_2 and HHT ($P < 0.05$) when assessed at the IC50 (**Table 3**). There was no significant inhibition at very low concentrations of EPA or 20:5 Δ17*t*, and a small range of fatty acid concentration around the IC50 produced a large variation in the extent of enzyme inhibition (see Fig. 2). When compared at the same concentrations, EPA always produced a greater inhibition than 20:5 Δ17*t*, but this effect decreased as the concentrations exceeded the IC50 of 20:5 Δ17*t*. To determine

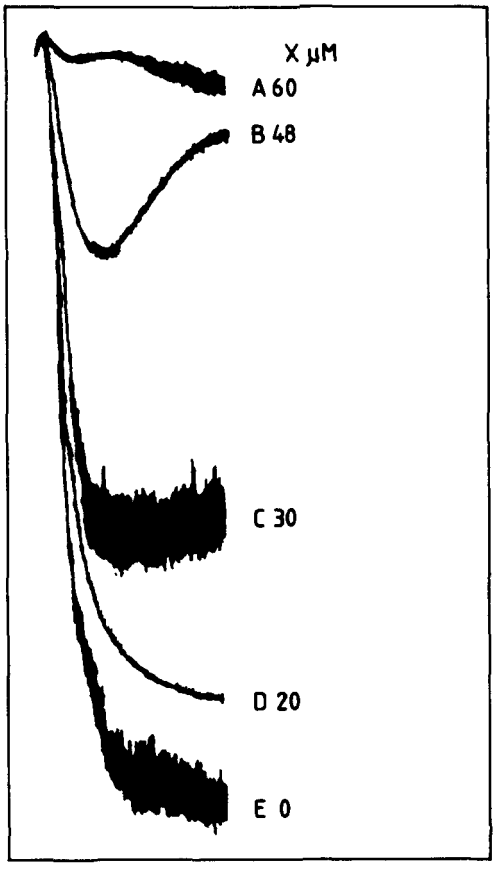

Fig. 1. Typical aggregation of human platelets stimulated by 2.5 μM 20:4 n-6. Amounts of 20:5 Δ17*t* added are shown beside aggregation trace.

TABLE 1. Purification scheme for 20:5 Δ*5c*, *8c*, *11c*, *14c*, *17t* from rat liver lipid

Purification Step	Weight %	Total mg	Fold Purification	Recovery %
Liver FAME	1.4	3,890	0	100
Prep HPLC	7.9	230	5.6	5.9
Semi-prep HPLC	62.0	190	44	4.9
$AgNO_3$ TLC	89.8	75.4	63	1.9
Analytical HPLC	99.3	47.6	70	1.2

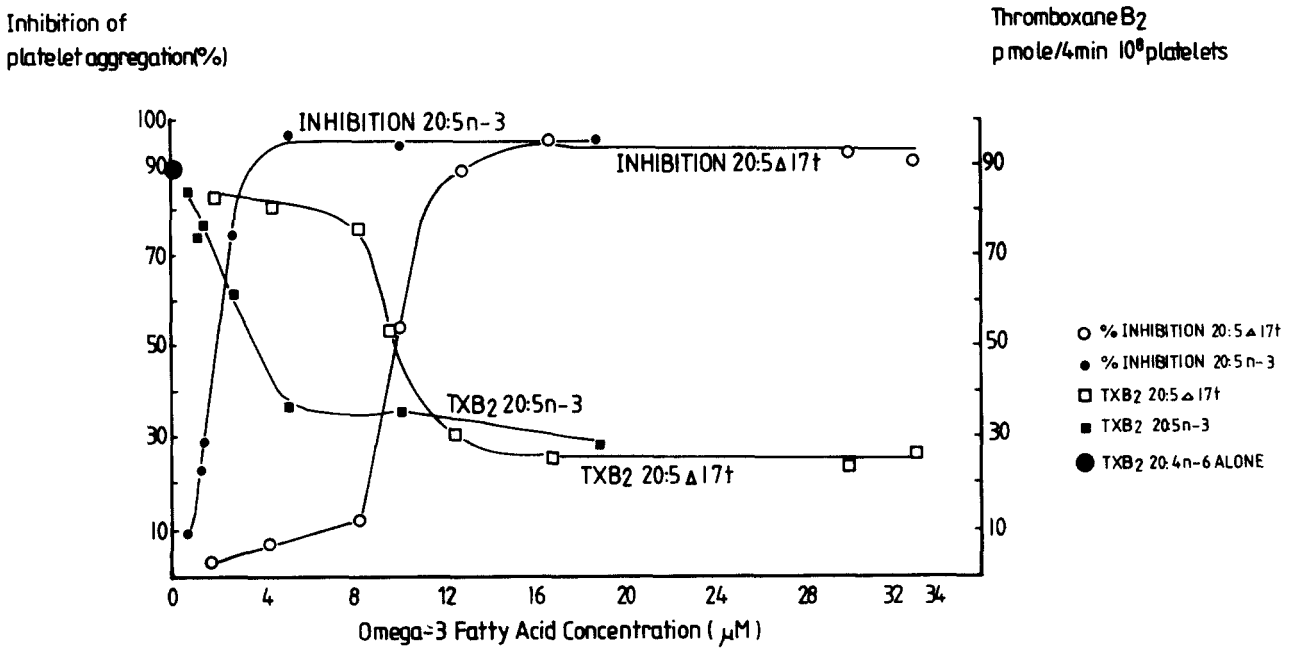

Fig. 2. Aggregation response of human platelets and thromboxane B_2 formation from 20:4 n-6 (2.5 μM) and in presence of 20:5 n-3 or 20:5 Δ17*t*.

whether the inhibition of aggregation by 20:5 Δ17*t* was due to inhibition of TXA_2 formation, the metabolite concentrations at the IC50 were calculated from linear regression of metabolite concentration versus % inhibition. This allowed comparison at equal activity and should show whether the inhibition patterns are similar, due to cyclooxygenase inhibition, and not an unknown nonspecific effect of the fatty acid. This was necessary in light of the observations by McIntyre et al. (23) who reported that *cis* unsaturated fatty acids, including oleic and linoleic, inhibited platelet aggregation in vitro in the concentration range 1–35 μM. The authors suggested that membrane perturbation was responsible for these observations. At the IC50, 20:5 Δ17*t* also significantly decreased the TXB_2 and HHT concentrations ($P < 0.05$) compared to AA control and there was no difference in the concentrations of cyclooxygenase metabolites in the presence of EPA or 20:5 Δ17*t* at their IC50s ($P > 0.05$).

It is interesting that 20:5 Δ17*t* did not induce platelet aggregation, although its structure, with a *trans* n-3 double bond, resembles AA. The mechanism of inhibition of platelet aggregation appears to be inhibition of TXA_2 formation for EPA and 20:5 Δ17*t*, although the concentrations necessary to produce the inhibition are different. One might hypothesize that the difference could arise from different binding affinities to cylooxygenase, different patterns of 20:5 metabolism, or membrane transport.

The level of AA incorporation into phospholipid was significantly lower in the presence of EPA or 20:5 Δ17*t* ($P < 0.05$) compared to the AA control. The lipoxygenase product HETE was higher than the AA control calculated at the IC50 in platelets incubated with 20:5 Δ17*t* but not with EPA. HETE formation is known to be peroxide tone dependent (24, 25). If 20:5 Δ17*t* is oxygenated by platelets, at least at high concentrations, its peroxide derivatives might provide such a required tone. The higher formation of HETE for 20:5 Δ17*t* calculated at the IC50 probably reflects the much higher concentrations used because there were no significant differences between EPA and 20:5 Δ17*t* when examined at similar, lower (5–20 μM) levels ($P > 0.05$, n = 6). This points out the

TABLE 2. N-3 fatty acid concentrations required to produce a 50% inhibition of platelet aggregation induced by 2.5 μM 20:4 n-6 (IC50)

	IC50 (μM)			
Replication	20:5 n-3	20:5 Δ17*t*	22:6 n-3	22:6 Δ19*t*
A	1.8	29.9	9.2	10.6
B	27.4	50.2	0.70	1.10
C	10.8	66.5	0.85	1.23
D	5.3	14.3	9.4	5.8
E	4.2	16.0	19.5	12.2
F	11.2	40.5	4.0	3.6
G	3.4	22.2	0.61	0.013
H	3.3	14.7	0.68	0.12
I	1.0	8.9		
Mean	7.6[a]	29.2[b]	5.6[a]	4.8[a]

Platelet samples were different for C20 and C22 n-3 fatty acids. Values having different superscripts are significantly different ($P < 0.05$).

TABLE 3. Effect of 20:5 isomers on C-20:4 n-6 (2.5 μM) metabolism by human platelets

	20:4 n-6 Control	20:4 n-6 + 20:5 n-3	20:4 n-6 + 20:5 Δ 17*t*
		pmol/10^8 platelets	
Phospholipid	67.3 ± 12.3[a]	46.6 ± 7.4[a]	33.5 ± 6.3[a]
Thromboxane B_2	42.0 ± 4.4[a]	32.8 ± 3.5[b]	30.6 ± 3.1[b]
HHT	91.9 ± 17.2[a]	68.3 ± 16.0[a,b]	65.8 ± 12.7[b]
HETE	49.4 ± 9.7[a]	50.6 ± 10.5[a]	108.8 ± 20.1[b]
Free 20:4 n-6	749.7 ± 30.3[a]	795.8 ± 30.3[b]	751.4 ± 26.5[a,b]

Data are calculated for concentration of 20:5 acids producing 50% inhibition of aggregation; mean ± SD. Values in rows having different superscripts are significantly different ($P < 0.05$).

need to compare these fatty acids over a range of concentrations.

The mean IC50s were 5.6 and 4.3 μM for DHA and 22:6 Δ19*t*, respectively. A comparison with the eicosapentaenoic acids is not valid because different blood samples were used for the two pairs of fatty acids and the variation within blood samples was quite large. A paired statistical test that removes or blocks the between-platelet sample variation is thus necessary to evaluate differences between fatty acids. This type of test cannot be performed for a comparison of data obtained from different blood samples.

Both DHA and 22:6 Δ19*t* significantly decreased cyclooxygenase-derived TXB_2 and HHT, compared to AA alone (**Table 4**). Furthermore, 22:6 Δ19*t* was a significantly better inhibitor of both metabolites compared to DHA when calculated at the IC50s ($P < 0.05$). TXB_2 was also significantly lower in the presence of 22:6 Δ19*t* compared to DHA when compared at equal concentrations (0.5–10 μM) and using two-way analysis of variance (to remove variation due to platelet preparations) whereas HHT approached significance at the 5% level (respective F-ratios: 11.03, 7.65; critical F0.05 1,4 df = 7.71). The parametric analysis of variance test was necessary because the two-tailed signed rank test at $P < 0.05$ is not applicable to sample sizes below 6. This contrasts with the data for the eicosapentaenoic acids where no significant differences were observed when calculated at the IC50s, and EPA was a significantly better inhibitor assessed at equal concentrations. HETE levels were not significantly different in the presence of either of the docosahexaenoic acids, in contrast with the enhanced level observed for 20:5 Δ17*t*.

The incorporation of AA into phospholipids, when added alone, appeared to be slightly better in the platelet preparations used for the EPA experiments compared to DHA (Tables 3, 4) although the difference was not significant ($P > 0.05$). The incorporation was decreased in the presence of either EPA or 20:5 Δ17*t* but not the C22 acids.

The metabolism of the n-3 isomers of EPA and DHA has not been examined. It is possible that this might explain some of the observations in addition to simple enzyme inhibition. Further studies are required to determine whether 20:5 Δ17*t* is metabolized to HEPE or TXA_3 analogs.

It appears that, in contrast to the results for EPA isomers, the *trans* double bond at the n-3 position in DHA does not alter the inhibitory effect on platelet aggregation. 22:6 Δ19*t* was even more effective in inhibiting platelet cyclooxygenase activity than DHA. The reason for this is still unclear

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TABLE 4. Effect of 22:6 isomers on C-20:4 n-6 (2.5 μM) metabolism by human platelets

	20:4 n-6 Control	20:4 n-6 + 22:6 n-3	20:4 n-6 + 22:6 Δ 19*t*
		pmol/10^8 *platelets*	
Phospholipid	44.4 ± 8.7[a]	45.3 ± 8.5[a]	48.5 ± 14.3[a]
Thromboxane B_2	42.6 ± 10.0[a]	27.6 ± 7.6[b]	22.7 ± 6.2[c]
HHT	111.4 ± 18.6[a]	65.1 ± 14.7[b]	48.1 ± 9.8[c]
HETE	83.6 ± 18.5[a]	102.6 ± 37.4[a]	90.2 ± 37.2[a]
Free 20:4 n-6	722.2 ± 43.4[a]	755.8 ± 71.0[a]	790.0 ± 49.4[a]

Data are calculated for concentration of 22:6 acids producing 50% inhibition of aggregation; mean ± SD. Values in rows having different superscripts are significantly different ($P < 0.05$).

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