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PREPARATION AND CHARACTERIZATION OF HYDROPEROXY-EICOSATETRAENOIC ACIDS (HPETEs)

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SUMMARY:

5-, 8-, 9-, 11-, 12- and 15 hydroperoxy-eicosatetraenoic acids (HPETEs) were generated from arachidonic acid by a reaction with H_2O_2 and Cu++ ions. They were purified by high performance liquid chromatography, either on a silica gel (μ Porasil) column or on a reversed phase (μ Bondapak C_{18}) column. The yield of 5-HPETE was considerably greater when the μ Bondapak C_{18} column was used. The HPETEs were characterized and assayed by their ability to oxidize triphenylphosphine: triphenylphosphine oxide formation was monitored by gas chromatography. When stored in methylene chloride at -20°C, the HPETEs were stable for several months.

INTRODUCTION:

There is a growing interest in the possible biological roles of hydroperoxy-eicosatetraenoic acids (HPETEs). 12-HPETE inhibits platelet thromboxane synthetase (1). 12- and 15-HPETEs block prostacyclin synthetase (2,3). 12-HPETE inhibits platelet cyclooxygenase, stimulates its own synthesis by a platelet lipoxygenase and prevents platelet aggregation (4). 12- and 15-HPETEs activate spleen guanylate cyclase (5). 15-HPETE enhances the release of anaphylactic mediators from the guinea pig lung (6). None of the biological actions cited above is reproduced by the corresponding hydroxy-acids. Furthermore, it was recently proposed that 5-HPETE is the precursor of a slow-reacting substance of anaphylaxis (7). Since a total chemical synthesis has not yet been developed for these compounds, the biological studies mentioned above were performed with materials prepared by biosynthesis (platelets for 12-HPETE, soybean lipoxidase for 15-HPETE). In this paper, we describe a convenient chemical method for generating multimilligram quantities of HPETEs, procedures for their purification, characterization and direct quantitation and data on their stability.

MATERIALS AND METHODS:

Preparation of HPETEs

Arachidonic acid (100 μ moles) and $CuCl_2$ (50 μ moles) were dissolved in methanol (10 ml) and the pH was brought to 7 by the addition of Tris buffer 0.2 M, pH 8.6 (2.5 ml). [H^3] arachidonic acid (2 to 10 μCi) was also added in order to monitor the subsequent

chromatography. H_2O_2 (3 mmoles, 0.2 ml of a 50% solution) was added. The reaction mixture was left for 30 min at room temperature and then extracted at pH 3 with 2 volumes of ethyl acetate. After solvent evaporation, the dry residue was dissolved in either 1 ml hexane-acetic acid (100:0.8,v/v) or 0.1 ml methanol, before the injection on the μ Porasil or the μ Bondapak C_{18} column, respectively.

High performance liquid chromatography

HPLC was performed with a Waters Associates Instrument (injector U6K, programmer model 600 and a model 6000A solvent delivery system). Two procedures were used. One involved a semi-preparative (7.8 mm X 30 cm) column of silica gel (μ Porasil, Waters Associates) and the delivery of a linear solvent gradient from hexane (with 0.8% acetic acid) to chloroform (with 0.8% acetic acid). Alternatively, a semi-preparative (7.8 mm X 30 cm) column of a reversed phase (μ Bondapak C_{18}, Waters Associates) was eluted isocratically with methanol-water-acetic acid (75:25:0.01, v/v) (8). After HPLC purification, HPETEs were stored in methylene chloride at -20°C, in concentrations of 0.2 to 0.6 mM.

UV spectrophotometry

UV absorption was measured, using a Beckman model 25 instrument.

Peroxide assay

A solution of triphenylphosphine in methanol was freshly prepared just prior to use. HPETEs (5-10 nmoles, according to UV absorption) were mixed with triphenylphosphine (13 nmoles): the reaction mixture was left at room temperature for 5 to 10 minutes. The solvent was then removed under a nitrogen stream and the dry residue was dissolved in methanol just prior to analysis by gas liquid chromatography. Gas chromatographic separation of triphenylphosphine and triphenylphosphine oxide was performed on a Varian 2100 instrument equipped with a flame ionization detector. A column of 3% OV-1 on Gas Chrom Q (2 m X 2 mm) was employed isothermally at 230°C.

Reduction of HPETEs

HPETEs were reduced with either $NaBH_4$ or triphenylphosphine, in methanolic solution. Following $NaBH_4$ reduction, the reaction mixture was diluted with water, acidified and extracted with ethyl acetate, before derivatization. Triphenylphosphine-reduced samples were directly derivatized, without prior extraction.

Derivatization

HETEs obtained from HPETEs by $NaBH_4$ reduction were esterified with ethereal diazomethane and silylated with a 1:1 mixture of bis-trimethylsilyl-trifluoro-acetamide (BSTFA) and pyridine. HETEs reduced by triphenylphosphine were converted to pentafluorobenzyl

(PFB) esters with a mixture of pentafluorobenzylbromide and diisopropylethylamine (9) and then silylated as described above.

Gas chromatography-mass spectrometry

Mass spectra were recorded using a Hewlett-Packard combined gas chromatograph-quadrupole mass spectrometer (model 5982A), operated in the electron ionization mode. Samples were injected on a 1 m X 2 mm column of 3% OV-1 on Gas Chrom Q with helium as carrier gas (flow rate: 30 ml/min). The temperature of the column was either 200°C (methyl esters) or 225°C (PFB esters); the injection port temperature was 250°C. Electron energy was 70 eV.

Materials

Arachidonic acid (> 99% pure) was purchased from Nu-Check Prep, Inc. [H^3]-arachidonic acid (60 Ci/mmole) was obtained from New England Nuclear. 50% H_2O_2 and triphenylphosphine were purchased from Fisher Scientific. The organic solvents for extraction and chromatography were obtained from Burdick and Jackson. 3% OV-1 on Gas Chrom Q was obtained from Applied Science Inc. BSTFA and pentafluorobenzylbromide were purchased from Pierce Chemical Company.

RESULTS:

Figure 1 compares the elution profile of the products of the reaction between arachidonic acid, Cu++ and H_2O_2 in two different chromatographic systems. HPETEs were identified according to the following criteria: the ultraviolet absorption spectrum, the retention indices in gas liquid chromatography and fragmentation patterns under electron impact of the methyl ester-trimethylsilyl ether (ME-TMS) or of the pentafluorobenzyl ester-trimethylsilyl ether (PFB-TMS) derivatives of the corresponding HETEs obtained by $NaBH_4$ or triphenylphosphine reduction and the oxidation of triphenylphosphine. UV spectrophotometry revealed an absorption maximum at 235 nm. The GC behavior (C-21.3, OV-1) and the fragmentation pattern of the ME-TMS derivatives of HETEs generated by this chemical procedure and $NaBH_4$ reduction are similar to those of HETEs of biological origin (10). Alternatively, HPETEs were reduced with triphenylphosphine and directly analyzed as PFB-TMS derivatives (C-25.8, OV-1). 5-HPETE, which had been purified by reversed phase HPLC, displayed major ions at m/z 572 (M), 482 (M-90, loss of trimethylsilanol), 421 (M-151, loss of $\cdot CH_2-(CH{=}CH-CH_2)_2-(CH_2)_3-CH_3$), 369 ($(CH_3)_3-SiO^+{=}CH-(CH_2)_3-COOCH_2C_6F_5$) and 305 (M-101, loss of $\cdot CH_2-(CH_2)_2-COOCH_2C_6F_5$). No ion characteristic of another HPETE was observed, except for 421 which is common to the 5- and 9- isomers. The proton NMR spectrum of 5-HETE was similar to that of 9-hydroxy-trans-10, cis-12-octadecadienoic acid (11).

HPETEs readily oxidized triphenylphosphine to triphenylphosphine oxide. These two compounds were easily separated by gas liquid

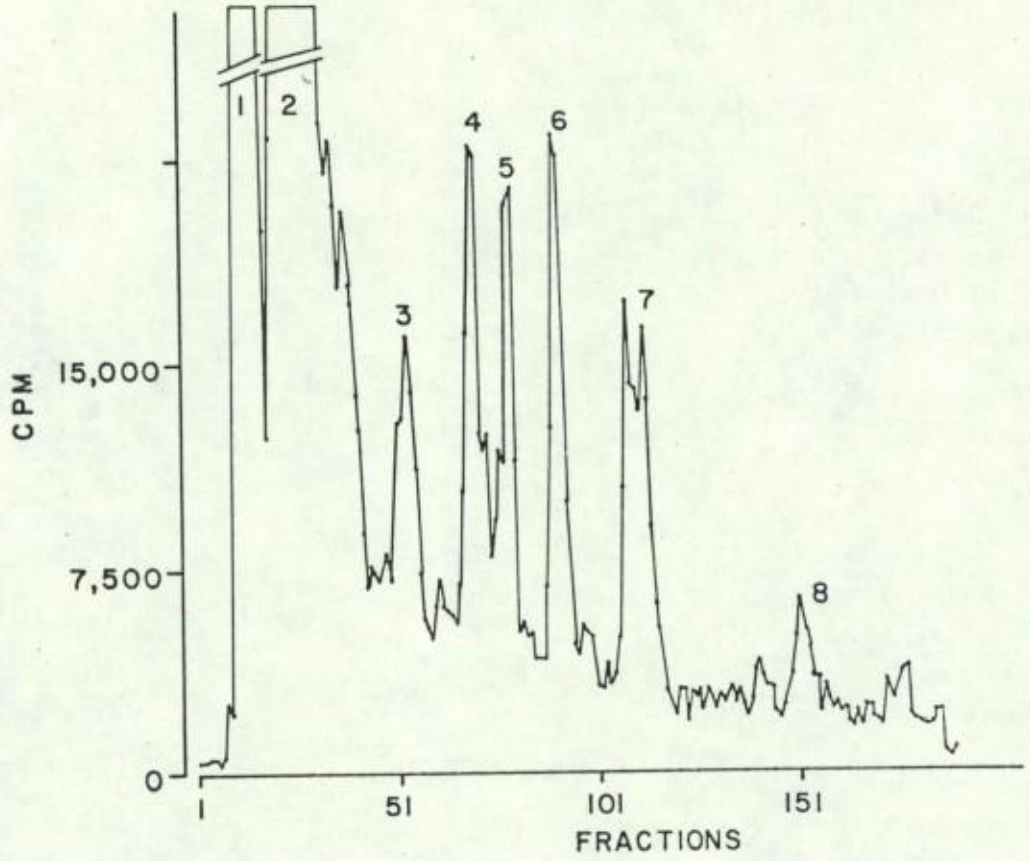

Figure 1 A. Separation of the products of the reaction between arachidonic acid, Cu++ and H_2O_2, by HPLC on a semi-preparative μ Porasil column (7.8 mm X 30 cm). A linear gradient from 10% to 50% chloroform-ethanol-acetic acid (100:1:0.8, v/v) in hexane-acetic acid (100:0.8, v/v) was delivered in 2 hours. The flow rate was 3 ml/min and 1.5 ml fractions were collected. Compounds were identified as described in results. 1: unreacted arachidonic acid; 2: uncharacterized compounds; 3: δ-lactone of 5-HETE; 4: 12-HPETE; 5: 15-HPETE; 6: 11-HPETE; 7: mixture of 8- and 9-HPETEs; 8: 5-HPETE.

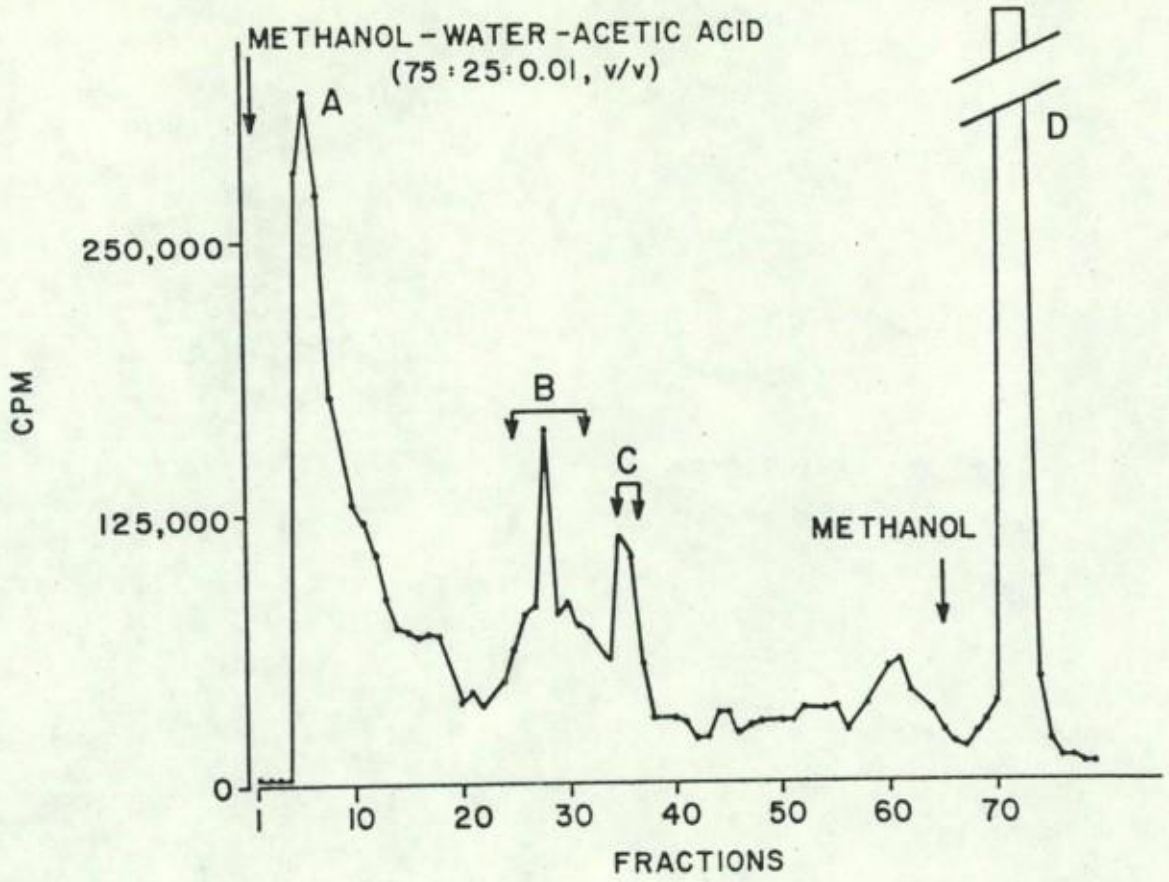

Figure 1B. Separation of the products of the reaction between arachidonic acid, Cu++ and H_2O_2, by HPLC on a semi-preparative μ Bondapak C_{18} column (7.8 mm X 30 cm). Elution was performed isocratically at a flow rate of 2.5 ml/min: 2.5 ml fractions were collected. Compounds were identified as described in results. A: uncharacterized compounds, B: mixture of 8-, 9-, 11-, 12-, 15-HPETEs (which were separated by a second HPLC on μ Porasil), C: 5-HPETE, D: unreacted arachidonic acid and uncharacterized compounds.

chromatography: as shown on figure 2, their equivalent chain lengths were C-18.4 and C-21.5, respectively (3% OV-1). The identity of the two GC peaks was confirmed by their mass spectra (12). The validity of the oxidation of triphenylphosphine as a method to assay HPETEs was established, using H_2O_2 as a standard. There was a 1:1 stoichiometric relationship between the amounts of H_2O_2 added and triphenylphosphine oxide produced (figure 3). We verified that HETEs were unable to oxidize triphenylphosphine. The blank of the assay could be minimized by avoiding exposure of triphenylphosphine solutions to air, as much as possible. Table 1 shows that in general a good correlation was observed between the concentrations of HPETEs determined by UV absorption and triphenylphosphine oxidation.

TABLE 1. Correlation between the concentrations of HPETEs, determined either as conjugated diene or as peroxide.

	CONJUGATED DIENE:mM (UV)	PEROXIDE:mM
8-+9-HPETEs	0.44 ± 0.001 (n=2)	0.54 ± 0.07 (n=10)
11-HPETE	0.35 ± 0.02 (n=2)	0.22 ± 0.03 (n=6)
12-HPETE	0.41 ± 0.01 (n=2)	0.52 ± 0.04 (n=9)
15-HPETE	0.27 ± 0.01 (n=2)	0.20 ± 0.04 (n=4)

Results express the mmolar concentrations of HPETEs (mean ± SD), measured by UV absorption (assuming $\varepsilon 235 = 27,000$) or by the oxidation of triphenylphosphine. HPETEs were purified by HPLC on μ Porasil (figure 1A). No loss of peroxide could be detected over the 4 months period during which repetitive determinations were performed (storage at -20°C in methylene chloride).

The concentration of 5-HPETE-purified on reversed phase-measured by the oxidation of triphenylphosphine was 102% ± 13% (mean ± SD: 3 preparations) of that determined by UV absorption.

The net yield of HPETEs recovered after HPLC on μ Porasil was 4% (mean of 2 preparations), but 5-HPETE accounted for only 0.2%. When the HPETEs were purified by reversed phase HPLC, the net yield of 5-HPETE was 1.1% ± 0.2% (mean ± SD: 3 preparations) (figure 1).

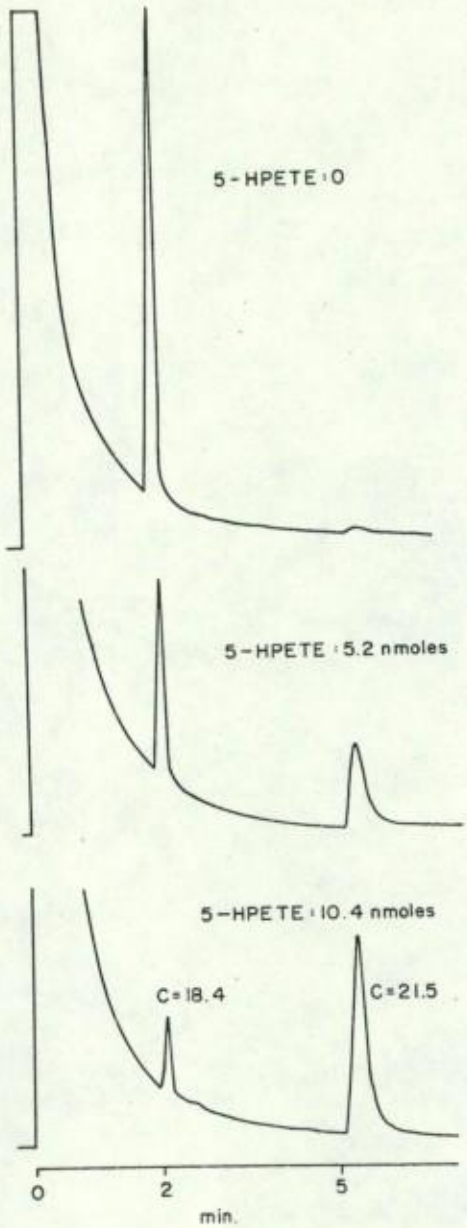

Figure 2. Identification and assay of 5-HPETE by the oxidation of triphenylphosphine. Various amounts of 5-HPETE, prepared by the reaction between arachidonic acid, Cu++ and H_2O_2 and purified by reversed phase HPLC, were mixed with triphenylphosphine (13 nmoles). Triphenylphosphine (C-18.4) and triphenylphosphine oxide (C-21.5) were separated by gas chromatography on a 3% OV-1 column at 230°C. The identity of the two peaks was confirmed by their mass spectra (12).

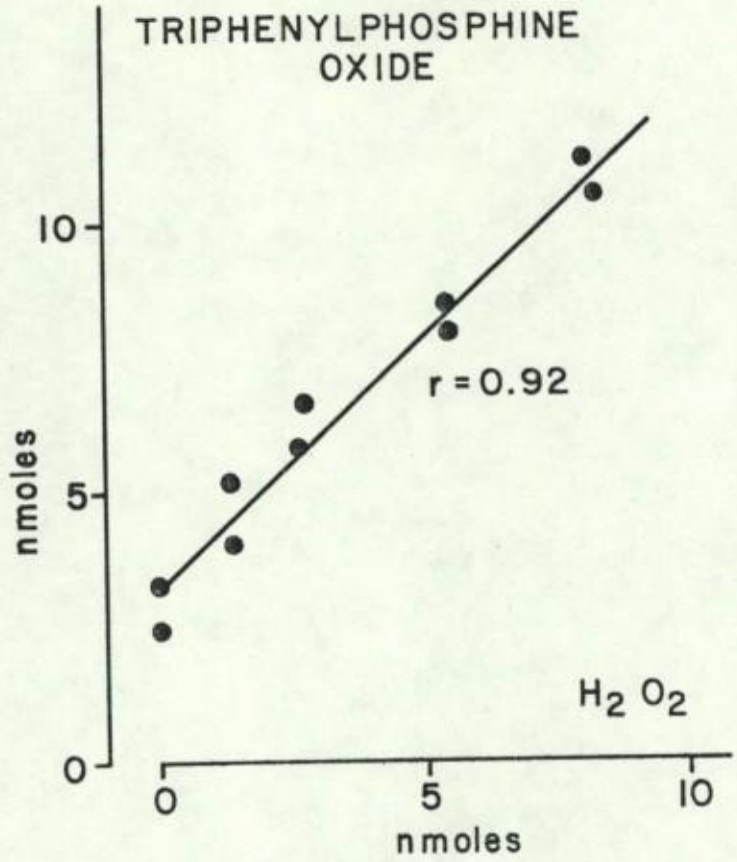

Figure 3. Stoichiometric relationship between peroxide added and triphenylphosphine oxide produced. In this experiment, the blank (amount of triphenylphosphine oxidized in the absence of peroxide) was quite high. It could be minimized by avoiding exposure to air as much as possible (see Figure 2: upper panel).

DISCUSSION:

The procedure for generation of HPETEs that we have developed is rapid, cheap and reproducible. The primary limiting factor is the time required to achieve HPLC purification of the products, since only limited quantities can be injected without overloading the columns used. Among the various HPETEs, the 5-isomer has a particular importance as the precursor of the leukotriene pathway (7). It could not be recovered in good yield from silica gel columns (μ Porasil). The yield was considerably increased by the use of a reversed phase column (μ Bondapak C_{18}) and the solvent system

described by Borgeat and Samuelsson (8). This difference in recovery might be related to the unique chemical reactivity of 5-HPETE.

The compounds generated by this procedure are identical to those of biological origin, by all available criteria, except for the probable lack of stereochemical purity. The presence of the UV absorption maximum at 235 nm and the GC retention times are compatible with a cis, trans configuration of the conjugated double bonds (10,11). In the case of the 5-HETE, NMR spectroscopy confirmed the existence of a trans double bond adjacent to the alcohol group and conjugated to a cis double bond (8,11).

The presence of a chromophore at 235 nm and the GC-MS behavior of reduction products do not provide an unambiguous demonstration that the products of the reaction, purified by HPLC, are HPETEs. The ability of these compounds to oxidize triphenylphosphine indicated the presence of a peroxide function and could be used for a direct assay of HPETEs. This assay is sensitive and accurate. It has a distinct advantage over the classical spectrophotometric assays of peroxides (13,14): the stoichiometric relationship between the quantity measured and the amount of peroxide added. As with other peroxide assays, it is essential to avoid exposure to air as much as possible in order to obtain a low blank.

When stored in methylene chloride at -20°C, HPETEs appeared quite stable: in solutions of 8-, 9-, 11-, 12- and 15-HPETEs, there was neither a detectable loss of peroxide, nor a shift of the UV absorption maximum from 235 to 232 nm (indicative of the isomerization of the conjugated diene from the cis-trans to the trans-trans configuration (11,15)) for at least 4 months. 5-HPETE was not degraded during purification at room temperature and was stable during storage at -20°C in methylene chloride for at least 2 weeks.

Recently, Porter et al. (15,16) described methods for generation and purification of HPETEs. 5-HPETE could be obtained after prolonged exposure of arachidonic acid to air (48 hours). Since it is known that hydroperoxides represent only a transient stage in the autooxidation of polyunsaturated fatty acids (13), the faster procedure described in this paper may be preferable. A comparison of the efficiency of the two procedures is not possible, since Porter et al. (15) did not specify the yield obtainable by autooxidation. Finally it must be mentioned that the HPETEs generated by either of these chemical methods are most likely racemic mixtures, in contrast with the stereospecificity of the lipoxygenase-catalyzed oxygenations (8,17,18).

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ABBREVIATIONS:

HPETE: hydroperoxy-eicosatetraenoic acid; HETE: hydroxy-eicosatetraenoic acid; HPLC: high performance liquid chromatography; GC-MS: gas chromatography-mass spectrometry; SD: standard deviation; r: regression coefficient; ME: methyl ester; PFB: pentafluorobenzyl ester; TMS: trimethylsilyl ether; C-: equivalent chain length.

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