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High-performance liquid chromatography–thermospray mass spectrometry of hydroperoxy polyunsaturated fatty acid acetyl derivatives

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ABSTRACT

A method for the analysis of hydroperoxy polyunsaturated fatty acids was developed. The hydroperoxy groups were acetylated by acetic anhydride, and the mixture was partially purified on a Sep-Pak C_{18} cartridge and analysed by high-performance liquid chromatography with thermospray mass spectrometry. Generally, the base ion, $[M + H - n(60)]^+$ or $[M + H - n(60) - n(H_2O)]^+$, is produced through elimination of acetic acid or water (n = number of hydroperoxy groups). The detection limit for these derivatives was *ca.* 1 pmol at concentrations of hydroperoxy polyenoic acids prior to derivatization. Using this method, many hydroxy and hydroperoxy polyunsaturated fatty acid derivatives could be detected simultaneously within 30 min on a selected-ion monitoring detection chromatogram without a gradient system. The assay was successfully applied to hydroxy and hydroperoxy polyunsaturated fatty acids from an incubation mixture of rat brain homogenate to which polyunsaturated fatty acids had been added.

INTRODUCTION

Almost all hydroperoxy polyunsaturated fatty acids (HPPUFAs) are produced via the lipoxygenase pathway from polyunsaturated fatty acids (PUFAs). HPPUFAs may be unstable [1] or immediately reduced after formation *in vivo* [2,3]. However, hydroperoxydocosahexaenoic acid (HPDHE) is sufficiently stable to permit analysis by high-performance liquid chromatography (HPLC) with UV detection [4,5] and HPDHE was produced from rat brain homogenate after incubation in room air for 1 h at 37°C [6].

HPPUFAs have been assayed by HPLC with UV detection [7] or electrochemical detection [3] and by gas chromatography–mass spectrometry (GC–MS) [8,9]. However, overlap in UV or electrochemical detection chromatograms and interference from other components in *in vivo* samples are not negligible [3,7]. The hydroperoxy group

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cannot not be detected directly by GC–MS because derivatization of the sample involved the chemical reduction of the hydroperoxy group to prevent decomposition of hydroperoxides on the GC column [8,9].

HPLC with thermospray (TSP) mass spectrometry has recently come to be used for analysis of labile polar biological samples since the spectrum of intact molecules can be readily obtained without prior derivatization. Kim and Salem [10] reported the determination of hydroperoxy derivatives of docosahexaenoic acid (DHE) and other polyunsaturates by HPLC–TSP-MS. However, this procedure is not sensitive enough, because fragments appeared following elimination of the hydroperoxy group.

We recently devised a new method for the determination of prostaglandin (PG)-related substances and hydroxy polyunsaturated fatty acids (HPUFAs) [11,12]. The hydroxyl groups were acetylated by acetic anhydride, partially purified on a Sep-Pak C_{18} cartridge and analysed by HPLC–TSP-MS. Generally, the base ion, $[M + H - n(60)]^+$, is produced through elimination of acetic acid (n = number of the hydroxyl group of the substance).

This paper describes a similar procedure in which the hydroperoxy groups of various HPPUFAs were acetylated, partially purified and analysed by HPLC TSP-MS. This procedure was successfully applied to HPPUFAs from an incubation mixture of PUFAs added to rat brain homogenate.

EXPERIMENTAL

Standards and reagents

HPPUFA standards 5(*S*)-hydroperoxyeicosatetraenoic acid [5(*S*)-HPETE], [12(*S*)-HPETE, 15(*S*)-HPETE, 5(*S*)-hydroperoxyeicosapentaenoic acid [5(*S*)-HPEPE], 15(*S*)-HPEPE and 9(*S*)-hydroperoxyoctadecadienoic acid [9(*S*)-HPODE] as well as 9(*S*)-hydroxyoctadecadienoic acid [9(*S*)-HODE] were obtained from Cascade Biochem (Reading, UK). 12-Hydroxy-[5,6,8,9,11,12,14,15-$^2H_8$]eicosa-5,8,10,14-tetraenoic acid [12(*S*)-HETE-d_8], 5(*S*)-hydroxyeicosapentaenoic acid [5(*S*)-HEPE] and 9(*S*)-HEPE were obtained from Cayman (Ann Arbor, MI, USA). *cis*-4,7,10,13,16,19-Docosahexaenoic acid (DHE) was obtained from Nu-Check Prep. (Elysian, MN, USA). *cis*-7,10,13,16,19-Docosapentaenoic acid (DPE) was obtained from Nacalai Tesque (Kyoto, Japan).

14-HDHE or 14-HDPE was enzymically prepared with DHE or DPE and the extract from human platelets as follows. Isolated human platelets (0.1 ml precipitated from the centrifuged suspension by 0.85% NaCl) were suspended in 22 ml of ice-cold 50 m*M* Tris–HCl buffer (pH 7.5) and sonicated at 20 kHz twice for 15 s. The sonicate was centrifuged at 2000 *g* for 20 min at 4°C. To 0.5 mg of DHE or DPE, 10 ml of the supernatant solution were added and the mixture was homogenized to disperse the substrate. The mixture was incubated at 20°C for 20 min in room air on a shaker operated at 120 rpm. The mixture was acidified to pH 3–4 with 2 *M* hydrochloric acid and applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed with 6 ml of water. The hydrophobic materials in the cartridge were eluted with 6 ml of acetonitrile, and the eluent was evaporated to dryness under reduced pressure. The residue was dissolved in 200 μl of acetonitrile and aliquots of the solution were applied to HPLC–TSP-MS with a mobile phase of 0.1 *M* ammonium formate–acetonitrile (2:5, v/v) as described below. The residual solution was applied to the HPLC system using the same column and mobile phase, with UV detection at 235 nm. The eluent of the UV absorbance peak corresponding to 14-HDHE or 14-HDPE was collected, diluted seven times with water, acidified to pH 3–4 with 2 *M* hydrochloric acid and applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed with 6 ml of water. 14-HDHE or 14-HDPE in the cartridge was eluted with 6 ml of acetonitrile, and the eluent was evaporated to dryness under reduced pressure.

14-Hydroxy-17-hydroperoxydocosahexaenoic acid (14-H,17-HPDHE), 14-hydroxy-17-hydroperoxydocosapentaenoic acid (14-H,17-HPDPE), 5-hydroxy-15-hydroperoxyeicosapen-

taenoic acid (5-H,15-HPEPE), 17-HPDPE or 17-HPDHE was enzymically prepared with 14-HDHE, 14-HDPE, 5(*S*)-HEPE, DHE or DPE and soybean lipoxygenase (LPX-1 code, Biozyme Labs., Blaenavon, Gwent, UK) as follows. A trace amount of the substrate and 14 000 U of soybean lipoxygenase in 1 ml of 0.2 *M* borate buffer, (pH 9.0) were vortex-mixed at room temperature for 1 min in room air. After addition of 2 ml of 0.2 *M* boric acid, the reaction mixture was acidified to pH 3–4 with 2 *M* hydrochloric acid, applied to the Sep-Pak C_{18} cartridge equilibrated with water, washed with 6 ml of water and eluted with 6 ml of acetonitrile. The eluent was evaporated to dryness under reduced pressure. 17-HPDHE or 17-HPDPE was purified on the HPLC–UV system and a Sep-Pak C_{18} cartridge as described above. 17-HDHE or 17-HDPE was prepared by the reduction of 17-HPDHE or 17-HPDPE by $NaBH_4$ as described previously [7].

Water for the HPLC eluent was of Milli-Q grade (Waters, Milford, MA, USA), that for preparing 5% acetonitrile, 2 *M* hydrochloric acid and 10% acetic acid, and for washing and equilibrating the Sep-Pak C_{18} cartridge (Waters) was prepurified by a Sep-Pak C_{18} cartridge. The other solvents and reagents were of analytical-reagent or chromatographic grade.

Extraction from rat brain homogenate

One male Wistar rat (weighing 150 g) was killed by decapitation, and its brain immediately excised at low temperature. The tissue (1.5 g of brain) was cut into pieces of *ca.* 2 × 2 mm and washed twice by decantation with 5 ml of 0.85% sodium chloride. The pieces were suspended in 25 ml of 50 m*M* Tris–HCl buffer (pH 7.52). The suspension was set on a nitrogen cavitation homogenizer (Parr Instrument, Moline, IL, USA) and homogenized after being subjected to nitrogen gas pressure at 100 kg/cm^2 for 20 min. Aliquots of DHE or DPE containing at *ca.* 900 nmol in ethanol were evaporated to dryness in each incubation tube under reduced pressure. Rat brain homogenate (5 ml each) was added, and the mixture was homogenized to disperse the substrate by a vortex-mixer. Each mixture was incubated at 37°C for 60 min on a shaker operated at 120 rpm. 12(*S*)-HETE-d_8 (608 pmol) as the internal standard (I.S.), 100 μl of 2 *M* hydrochloric acid, 0.25 ml of ethanol and 5 μl of butylated hydroxytoluene (BHT) ethanol solution (50 mg/ml) were added. Each mixture was centrifuged at 1500 *g* for 10 min at 4°C to remove excess tissue, and each supernatant was applied to a Sep-Pak C_{18} cartridge equilibrated with water. The cartridge was washed with 10 ml of water. HPPUFAs and HPUFAs in the cartridge were eluted with 6 ml of acetonitrile, and the eluent was evaporated to dryness under reduced pressure.

Derivatization

HPPUFAs and HPUFAs were derivatized using acetic anhydride in pyridine to obtain the acetic ester, and the acetyl derivatives of HPPUFAs and HPUFAs were partially purified on a Sep-Pak C_{18} cartridge as previously described [11,12]. The isolated HPPUFA and HPUFA fractions were dissolved in 200 μl of acetonitrile, and 20-μl aliquots were subjected to HPLC–TSP-MS.

HPLC–TSP-MS

A Shimadzu (Kyoto, Japan) LC/GC/MS-QP 1000S, equipped with a Vestec (Houston, TX, USA) Model 750B HPLC–TSP-MS interface, Shimadzu LC-9A HPLC pump and Rheodyne injector fitted with a 20-μl loop, was used. HPLC separation was carried out using a Nucleosil 100 $5C_{18}$ column (5 μm particle size, 150 mm × 4.6 mm I.D., Macherey Nagel, Duren, Germany), with a mobile phase of 0.1 *M* ammonium formate–0.1 *M* formic acid–acetonitrile (8:2:15, v/v) at a flow-rate of 1.0 ml/min. A mobile phase of 0.1 *M* ammonium formate–acetonitrile (2:5, v/v) was used for HPLC–TSP-MS of HPPUFAs and HPUFAs prior to derivatization.

The TSP interface temperature was optimized for maximum detection sensitivity for the acetyl derivatives. In the positive-ion mode, the optimal vaporizer control, vaporizer tip, vapour, block and tip heater temperatures were maintained at 146, 280, 323, 346 and 344°C, respectively, under electron beam-off or electrical discharge-off conditions. A new vaporizer has been made highly

TABLE I

RELATIVE ABUNDANCES OF IONS IN THE TSP SPECTRA OF HPPUFA OR HPUFA ACETYL DERIVATIVES

HPPUFA OR HPUFA	Acetyl group	M	[M + Na]	[M+Na −H2O]	[M+NH4]	[M+NH4 −H2O]	[M+H]	[M+H −H2O]	[M + Na −60]
9(*S*)-HODE	1	338	6	–	–	–	–	–	–
9(*S*)-HPODE	1	354		–	–	1	–	–	10
5(*S*)-HPETE	1	378		–	–	–	–	–	45
12(*S*)-HPETE	1	378	–	73	–	42	–	11	19
15(*S*)-HPETE	1	378	9	–	–	–	25	–	59
5(*S*)-HPEPE	1	376	–	–		–	5	–	96
15(*S*)-HPEPE	1	376	–	47	–	25	–	3	–
17-HDPE	1	388	6	–		–	–	–	–
17-HPDPE	1	404	–	12		15	6	2	4
17-HDHE	1	386	10	–	–		–	–	–
17-HPDHE	1	402	3	40	–	42	13	7	13
5,15-DiHEPE	2	418	–	–	–	–			11
5-H,15-HPEPE	2	434	–	–	–	–	–	2	3
14,17-DiHDPE	2	446	–	–	–	–	–	–	46
14-H,17-HPDPE	2	462	–	41	–	62	–	–	–
14,17-DiHDHE	2	444	–	–	–	–	–	–	83
14-H,17-HPDHE	2	460	–	–	–	1	–		1

sensitive by running with a mobile phase of 0.1 *M* ammonium acetate–0.1 *M* acetic acid–acetonitrile (4:6:15, v/v) under the temperature conditions described above.

RESULTS AND DISCUSSION

In the incubation mixture of DHE or DPE and the extract from human platelets under the experimental conditions, the main product was 14-HDHE or 14-HDPE. Yields calculated from the UV absorbance of 14-HDHE or 14-HDPE at 235 nm indicated *ca.* 0.6 or 1.2% of DHE or DPE to have been converted, respectively. In the incubation mixture of DHE or DPE and soybean lipoxygenase under the experimental conditions, the main product was 17-HPDHE or 17-HPDPE. Yields calculated from the UV absorbance of 17-HPDHE or 17-HPDPE at 235 nm indicated *ca.* 38 or 52% of DHE or DPE to have been converted, respectively. Yields calculated from the UV absorbance of 17-HDHE or 17-HDPE at 235 nm indicated *ca.* 65 or 41% of 17-HPDHE or 17-HPDPE to have been converted, respectively.

MS patterns of derivatives each showed a characteristic base ion (Fig. 1). As shown in Table I, the base ion was $[M + H - n(60)]^+$ or $[M + H - n(60) - n(H_2O)]^+$, based on the elimination of acetic acid (60 mass units) or water from the molecular ion. The rates of ion counts of $[M + H - 60]^+$ and $[M + H - 60 - H_2O]^+$ compared with those of the corresponding peak in the total ion chromatogram (TIC) from the 17-HPDHE derivative in Fig. 1D were 32 and 29%, respectively. This parameter for $[M + H - 60]^+$ against that of the corresponding peak in TIC from 17-HDHE derivative in Fig. 1E was 69%. Chemical ionization due to an electric beam (filament on 150 μA) or electrical discharge caused no increase in the ion intensity of the acetyl derivatives. A peak component that eluted earlier than the derivative of 17-HPDPE or 17-HPDHE in Fig. 1C (*m/z* 345, 367) or Fig. 1D (*m/z* 343, 365) faintly caused elimination of water, and thus this compound was considered to be an epoxy-hydroxy-DPE or epoxy-hydropxy-DHE, as described previously [10]. The formation of these compounds may possibly result from rearrange-

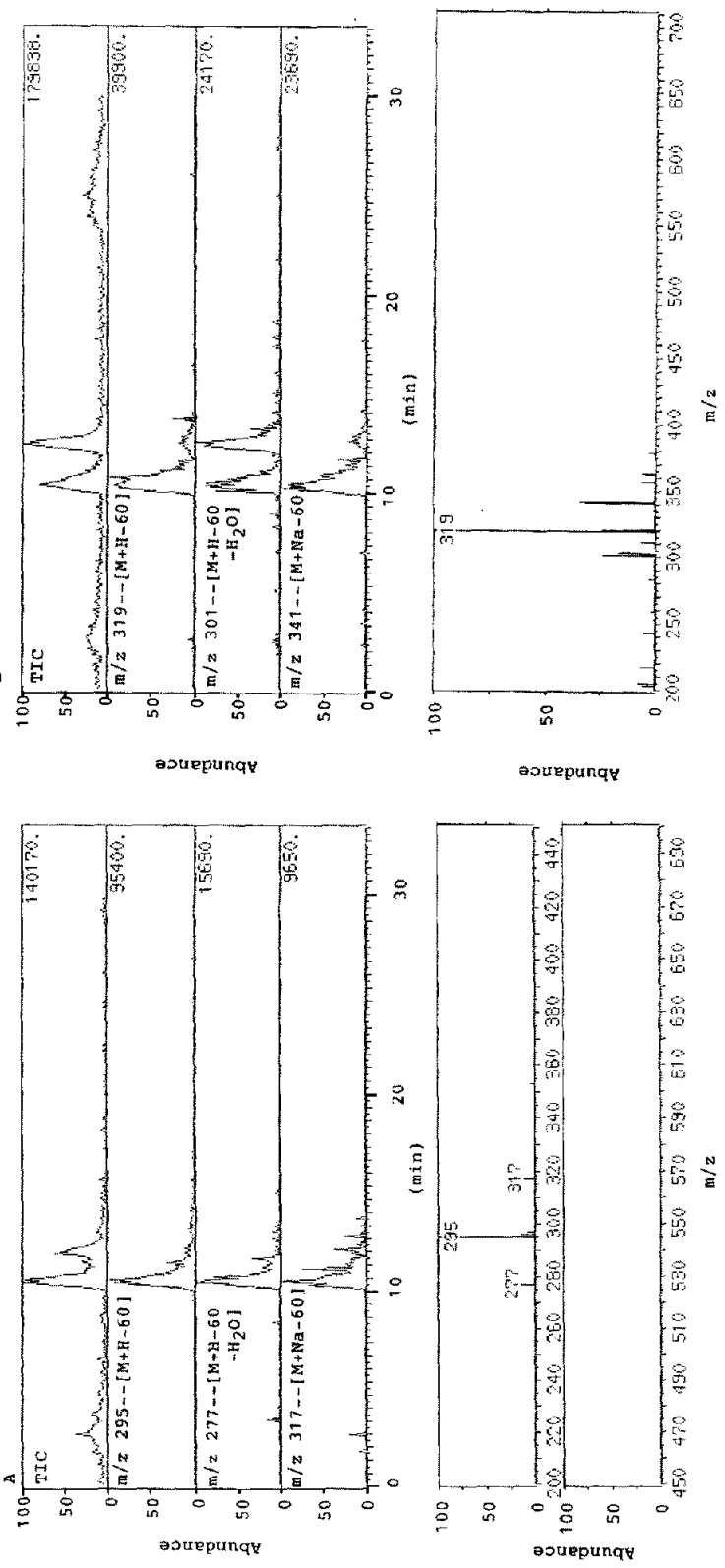
[M+Na −60−H_2O]	[M+NH_4 −60]	[M+NH_4 −60−H_2O]	[M+H −60]	[M+H−60 −H_2O]	[M+Na −120]	[M+Na −120−H_2O]	[M+NH_4 −120]	[M+NH_4 −120−H_2O]	[M+H −120]	[M+H−120 −H_2O]
–	–	–	100	–						
–	4	1	100	16						
–	1	–	100	12						
–	11	5	100	71						
–	3	1	100	30						
–	–	3	100	23						
–	–	–	48	100						
–	–	–	100	–						
–	4	4	51	100						
–	8	–	100	–						
–	10	6	100	89						
–	–	–	–	–	–	–	–	–	100	–
29	–	39	17	24	–	–	15	11	67	100
–	45	–	–	–	–	–	–	–	100	–
–	–	–	–	–	–	–	–	75	100	71
–	–	–	–	–	–	–	–	–	100	–
–	–	–	1	–	–	–	–	1	100	90

ment of the hydroperoxy group, as has been noted for 12-HPETE [13].

A peak appearing at a retention time of 11–13 min in the TIC of Fig. 1 gave a base ion of *m/z* 279. The peak was obtained even in a blank experiment, and thus was assigned as an unidentified component unrelated to HPPUFAs or HPPUFAs. This *m/z* 279 component may possible have resulted from the byproducts of the acetylation. Since peaks corresponding to unreactive HPPUFAs are very few in the TIC of Fig. 1, the conditions used are adequate for the complete acetylation of each HPPUFA. However, the sub-ion of *m/z* 279 component appeared in the area of *m/z* 301, 319, 341 and 345, as shown in Fig. 1B and C.

Acetyl derivatives of HPPUFAs and their reduction products could be easily detected by selected-ion monitoring (SIM), as shown in Fig. 2. Fig. 3 shows an SIM chromatogram of HPPUFAs and HPUFAs prior to derivatization of *ca.* 1 pmol. The limit of detection was *ca.* 0.5–1 pmol.

SIM chromatograms of acetyl derivatives of extracts from an incubation mixture of PUFA additional rat brain homogenate are shown in Figs. 4–6. On chromatograms from an additional DHE homogenate, peaks due to derivatives of 5(*S*)-HETE, 9(*S*)-HETE, 15(*S*)-HETE, 17-HPDHE, 16-HDHE, 17-HDHE, 14-HDHE, 17HDPE, epoxy-hydroxy-DHE, 14,17-DiHDPE and 14,17-DiHDHE could be seen (Fig. 4). Similarly, 5(*S*)-HETE, 9(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 16-HDPE, 17-HDPE, 14-HDPE, 16-HDHE, 17-HDHE, 14-HDHE and epoxy-hydroxy-DPE from additional DPE homogenate (Fig. 5), 5(*S*)-HETE, 9(*S*)-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 16-HDHE, 17-HDHE, 14-HDHE, 17-HPDHE and 14,17-DiHDHE from non-additional homogenate could be seen (Fig. 6). By comparing HETE derivatives (*m/z* 303) with HDHE derivatives (*m/z* 327) or HDPE derivatives (*m/z* 329) in Figs. 4–6, the addition of DHE or DPE was shown not to inhibit the biosynthesis of HETEs. As described in a previous paper [12], the addition of homo-γ-linolenic acid ($n - 6$), arachidonic ($n - 6$) or eicosapentaenoic acid ($n - 3$) to rat brain homogenate caused no inhib-


A
TIC
140170.
m/z 295--[M+H-60]
95490.
m/z 277--[M+H-60 -H2O]
15650.
m/z 317--[M+Na-60]
9650.
Abundance
(min)
B
TIC
179630.
m/z 319--[M+H-60]
39300.
m/z 301--[M+H-60 -H2O]
24170.
m/z 341--[M+Na-60]
29690.
Abundance
(min)
319
m/z
Abundance
277
295
317
m/z

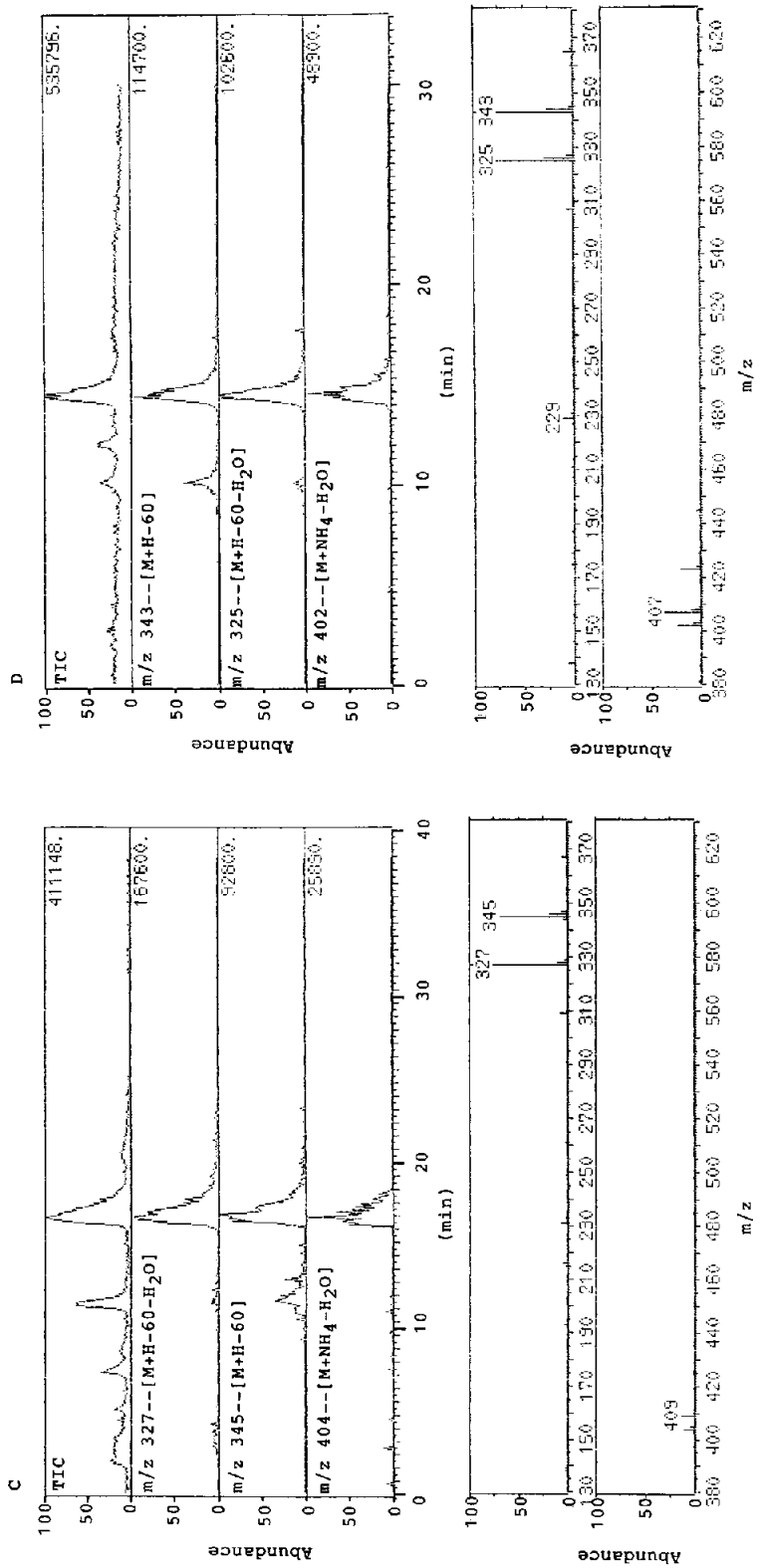


Fig. 1.

(Continued on p. 32)

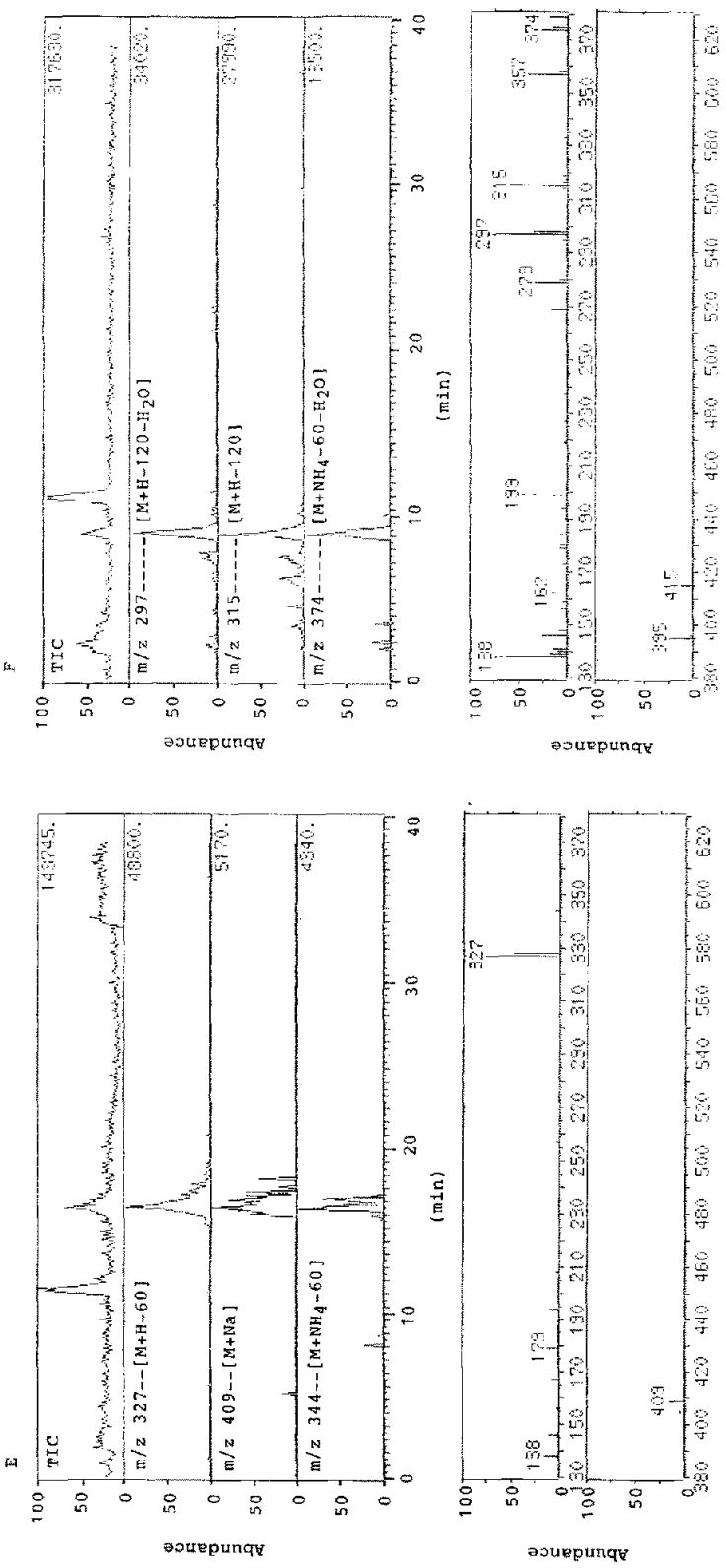


Fig. 1. TIC profile, mass chromatogram profile on each *m/z* number and mass spectra obtained from HPPUFA or HPUFA acetyl derivatives. HPLC and TSP conditions as described in Experimental. Scan speed, 1.0 scan/s from *m/z* 130 to 630. Filament off. Spectral patterns corresponding to the main peak on TIC are shown below. (A) 9(S)-HPODE acetyl derivative; (B) 15(S)-HPETE acetyl derivative; (C) 17-HPDPE acetyl derivative; (D) 17-HPDHE acetyl derivative; (E) 17-HDHE acetyl derivative; (F) 5-H,15-HPEPE acetyl derivative. The number in the upper right-hand corner of each chromatogram is the ion count.

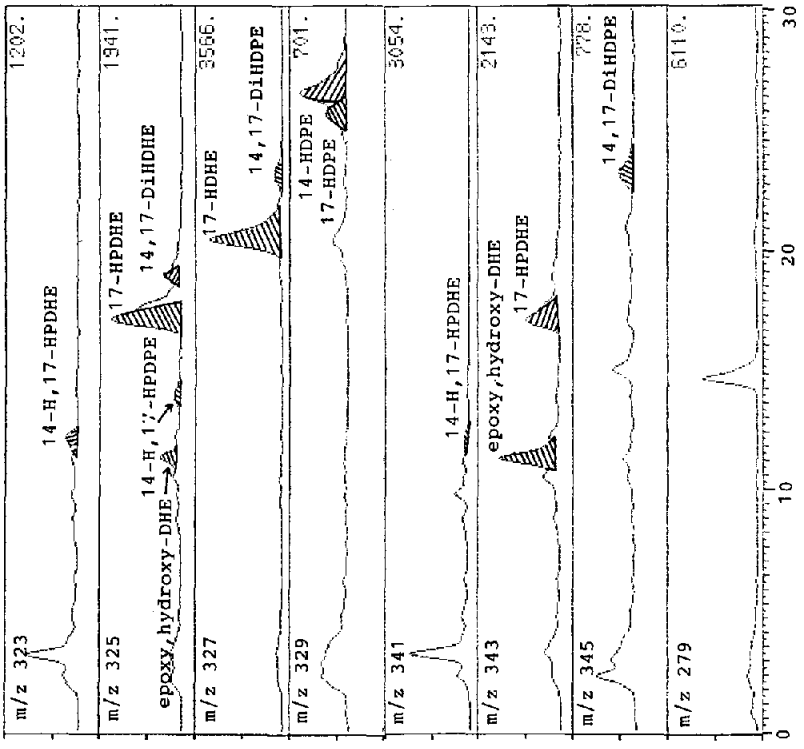


Fig. 2. SIM chromatogram of acetyl derivatives of HPPUFAs and their reduction products at *ca.* 5–20 pmol prior to derivatization. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

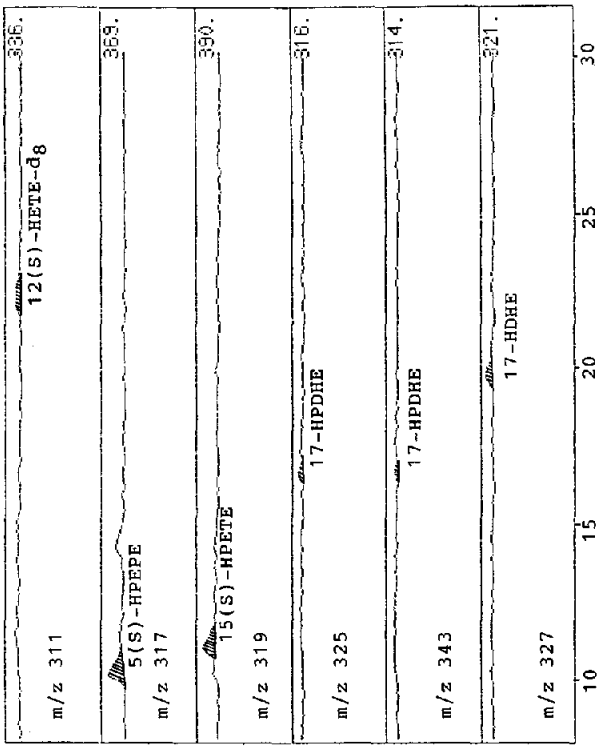


Fig. 3. SIM chromatogram of acetyl derivatives of HPPUFAs and HPUFAs at *ca.* 1 pmol prior to derivatization. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

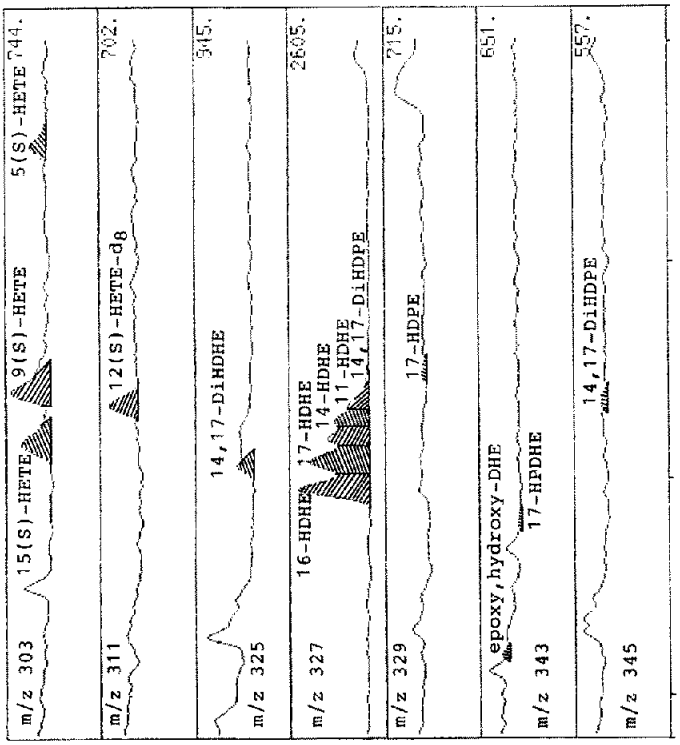


Fig. 4. SIM chromatogram of acetyl derivatives of extracts from an incubation mixture of DHE additional rat brain homogenate. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

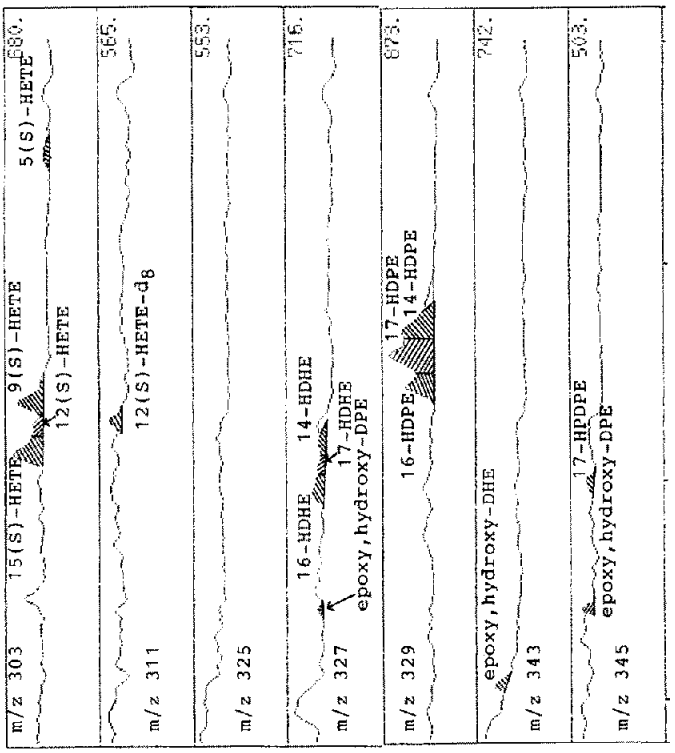


Fig. 5. SIM chromatogram of acetyl derivatives of extracts from an incubation mixture of DPE additional rat brain homogenate. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

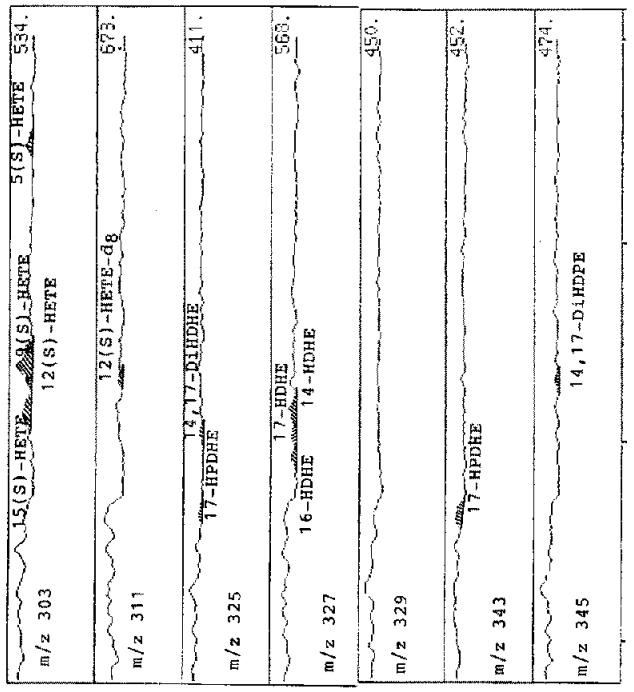


Fig. 6. SIM chromatogram of acetyl derivatives of extracts from an incubation mixture of non-additional rat brain homogenate. HPLC and TSP conditions as described in Experimental. The number in the upper right-hand corner of each chromatogram is the ion count.

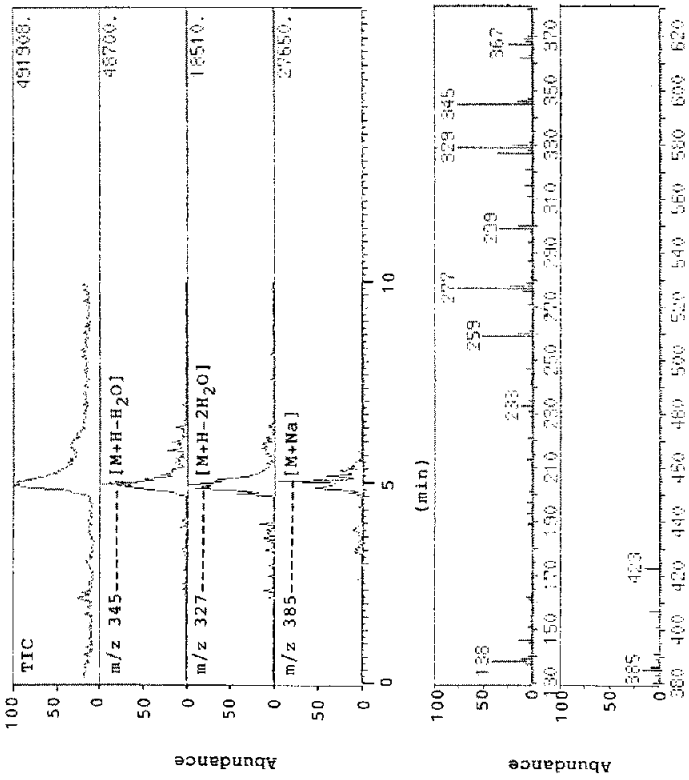


Fig. 7. TIC profile, mass chromatogram profile on each *m/z* number and mass spectra obtained from underivatized 17-HPDPE. HPLC and TSP conditions as described in Experimental. Scan speed, 1.0 scan/s from *m/z* 130 to 630. Filament off. Spectral patterns corresponding to the main peak on TIC are shown below. The number in the upper right-hand corner of each chromatogram is the ion count.

ition of the biosynthesis of HDHEs. Lipoxygenase-selecting eicosaenoic acid as substrate would thus not appear to be the same as lipoxygenase-selecting docosaenoic acid as substrate.

HPLC–TSP-MS techniques were used previously to determine HPPUFAs [10], but the sensitivity was inadequate because different fragment ions appeared following the elimination of the hydroxy groups (Fig. 7).

However, by the present method, hydroxy groups of HPPUFAs or HPUFAs are acetylated and base ions $[M + H - n(60)]^+$ or $[M + H - n(60) - n(H_2O)]^+$ in the mass spectra are thus strong without much fragmentation (Table I). The sensitivity of HPPUFAs is much improved without negative ionization or the filament mode. The derivatives are characterized by a limited range of polarity, and thus the HPLC retention times of HPPUFAs derivatives are only 30 min when a single mobile phase is used and detection is by TSP-MS. In this method, the mobile phase composition on the TSP interface is fixed because a single mobile phase is used, and this lessens the variation in sensitivity.

This method permits the simultaneous analysis of all HPPUFAs by HPLC–TSP-MS and should thus be applicable to the study of the biosynthetic metabolism of HPPUFAs.

REFERENCES

1 H. Frank, M. Wiegand, M. Strecker and D. Thiel, *Lipids*, 22 (1987) 689.
2 M. VanRollins and R. C. Murphy, *J. Lipid Res.*, 25 (1984) 507.
3 J. Terao, S. S. Shibata and S. Matsushita, *Anal. Biochem.*, 169 (1988) 415.
4 H. Hughes, C. V. Smith, E. C. Horning and J. R. Mitchell, *Anal. Biochem.*, 130 (1983) 431.
5 K. A. Ansari and D. Shoeman, *J. Chromatogr.*, 439 (1988) 453.
6 K. A. Ansari and D. Shoeman, *J. Chromatogr.*, 475 (1989) 457.
7 G. Graff, L. A. Anderson and L. W. Jaques, *Anal. Biochem.*, 188 (1990) 38.
8 H. W. S. Chan and F. A. A. Prescott, *Biochim. Biophys. Acta*, 380 (1975) 141.
9 H. Hughes, C. V. Smith, J. O. Tsokos-Kuhn and J. R. Mitchell, *Anal. Biochem.*, 152 (1986) 107.
10 H.-Y. Kim and N. Salem, Jr., *Prostaglandins*, 37 (1989) 105.
11 M. Yamane and A. Abe, *J. Chromatogr.*, 568 (1991) 11.
12 M. Yamane and A. Abe, *J. Chromatogr.*, 575 (1992) 7.
13 C. R. Pace-Asciak, *J. Biol. Chem.*, 259 (1984) 8332.