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Enantiomeric separation and analysis of unsaturated hydroperoxy fatty acids by chiral column chromatography-mass spectrometry

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ABSTRACT

Hydroperoxides of 18:2n-6 and 20:4n-6 were obtained by autoxidation and photooxidation. The enantiomers were separated as free acids (Reprosil Chiral-NR column, eluted with hexane containing 1–1.2% alcoholic modifier) and analyzed by on line UV detection (234 nm) and liquid chromatography-MS/MS/MS of carboxylate anions ($A^- \rightarrow (A^- - 18) \rightarrow$ full scan) in an ion trap. The combination of UV and MS/MS/MS analysis facilitated identification of hydroperoxides even in complex mixtures of autoxidized or photooxidized fatty acids. The signal intensities increased about two orders of magnitude by raising the isolation width of A^- from 1.5 amu to 5 or 10 amu for *cis-trans* conjugated hydroperoxy fatty acids, and one order of magnitude or more for non-conjugated hydroperoxy fatty acids. The *S* enantiomer of 8-, 9-, 10-, and 13-hydroperoxyoctadecadienoic acids and the *S* enantiomer of *cis-trans* conjugated hydroperoxyeicosatetraenoic acids eluted before the corresponding *R* enantiomer with two exceptions (11-hydroperoxylinoleic acid and 8-hydroperoxyeicosa-5*Z*,9*E*,11*Z*,14*Z*-tetraenoic acid). The separation of enantiomers or regioisomers could be improved by the choice of either isopropanol or methanol as alcoholic modifier.

1. Introduction

Hydroperoxides of polyunsaturated fatty acids can be formed non-enzymatically and by dioxygenases of animals, plants and fungi [1,2]. Lipoxygenases are non-heme iron or manganese enzymes, which transform polyunsaturated fatty acids to *cis-trans* conjugated hydroperoxides [1,3]. The hydroperoxides are often formed with a high degree of stereo and position specificity. The prototype plant lipoxygenase is soybean lipoxygenase-1, whereas arachidonate 5-, 12-, and 15-lipoxygenases occur in humans and animals [1]. The human 5-lipoxygenase is of particular medical interest, as it forms leukotriene A_4, an precursor to important mediators of allergic inflammation and bronchial asthma, the cysteinyl leukotrienes [4]. With only few exceptions, plant and mammalian lipoxygenases form hydroperoxides with *S*-stereoconfiguration. Lipoxygenases forming hydroperoxides with *R*-stereoconfiguration occur in marine organisms [5], human [6] and mouse skin [7], and in the Take-all fungus, *Gaeumannomyces graminis* [3]. Hydroperoxides can also be formed by heme-containing fatty acid dioxygenases, *viz.* cyclooxygenases, linoleate diol synthases, linoleate 10*R*-dioxygenase and plant α-dioxygenases [1,2,8]. Steric analysis of produced hydroperoxides has emerged as an essential tool for studies of important amino acid residues in the active site for substrate positioning and catalysis [7,9–12].

The stereoconfiguration of fatty acid hydroperoxides was first determined by chemical methods, e.g., chemical degradation, derivatization, ozonolysis and separation of diastereoisomers by gas chromatography–mass spectrometry. Using standards of known configuration, chiral phase HPLC has been used for steric analysis of hydroxy fatty acids. Enantiomeric separation of *cis-trans* conjugated hydroxyeicosatetraenoic and hydroxyoctadecadienoic methyl esters was first reported on columns with dinitrobenzoyl α-phenylglycine [13]. At the same time, a series of chiral columns were developed based on a matrices with derivatized amylose or cellulose (e.g., Chiralpak and Chiralcel series[1]), and used for steric analysis of *cis-trans* conjugated hydroxy fatty acids [7,14–16]. There is less information on separation of hydroperoxy fatty acids. The enantiomers of methyl ester derivatives of hydroperoxyeicosatetraenoic acids (HPETEs) were recently separated by chromatography on Chiralpak AD (amylose tris(3,5-dimethylphenylcarbamate coated on silica) in the reversed phase mode with excellent resolution of the enantiomers [17]. Unfortunately, the analytical Chiralpak and Chiralcel HPLC columns have

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[1] Home page (www.esslab.com/daicel_chiral_columns.htm).

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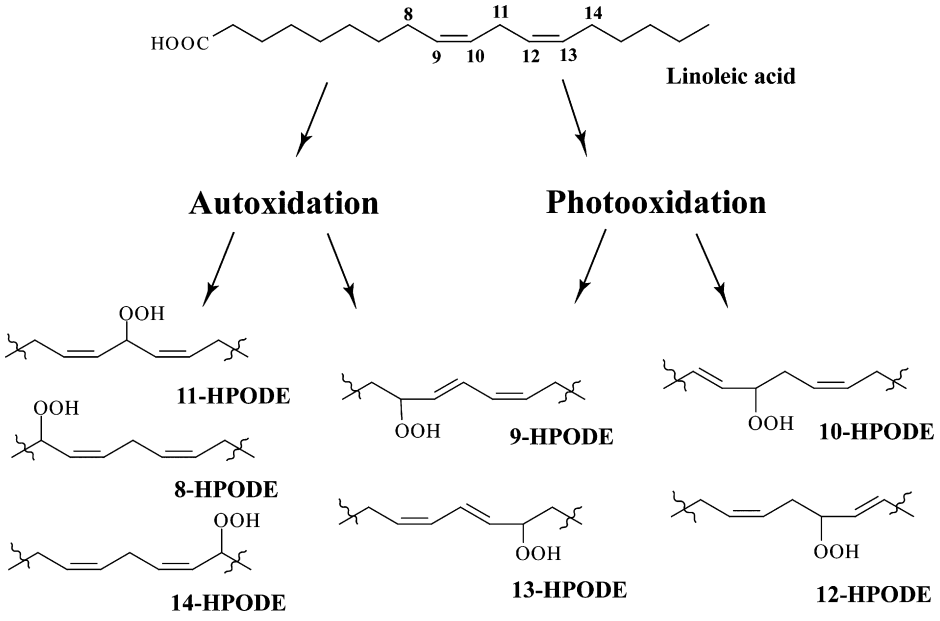

Fig. 1. Overview of hydroperoxides of 18:2n-6 formed by autoxidation and photooxidation. A majority of these hydroperoxides can also be formed by dioxygenases. Lipoxygenases can form 9-, 11-, and 13-HPODE and heme-containing dioxygenases 8- and 10-HPODE.

two draw-backs. First, they are very expensive[1]. Second, the immobilized polysaccharide matrices can only be operated at relatively low pressures.

The Reprosil Chiral-NR columns contain silica with a covalently bound chiral aromatic selector[2]. In many cases, separations are comparable to matrices based on polysaccharides with chiral selectors discussed above. Unfortunately, the chiral selector of Reprosil Chiral-NR is not disclosed. Aromatics with oxygen or nitrogen near the chiral center can often be separated, but we found that Reprosil Chiral-NR also could be used to separate the enantiomers of 10-hydroxyoctadeca-8*E*,12*Z*-dienoic acid (10-HODE) as free acids [18]. This prompted us to study whether this column could be used for enantiomeric separation of hydroperoxides of 18:2n-6 and *cis-trans* conjugated hydroperoxides of 20:4n-6 as free acids.

There are two reasons to separate and isolate hydroperoxides of 18:2n-6 and 20:4n-6 as free acids. First, hydroperoxides can be enzymatically transformed but not their methyl esters. For example, (8*R*)-hydroperoxyoctadecadienoic (8*R*-HPODE) can be isomerized to dihydroxy fatty acids by fungal diol synthases and (12*R*)-hydroperoxyeicosa-5*Z*,8*Z*,10*E*,14*Z*-tetraenoic acid (12*R*-HPETE) to epoxyalcohols by epidermal lipoxygenase-3 in human skin [2,19,20]. Second, hydroperoxy fatty acids can be detected and conveniently identified by negative ion electrospray ionization mass spectrometry [21,22]. This is of particular value for hydroperoxides without *cis-trans* configuration (and without UV absorbance at 234 nm). An overview of hydroperoxides of 18:2n-6, which are formed by autoxidation and photooxidation, is shown in Fig. 1. A majority of these hydroperoxides can also be formed by dioxygenases.

In recent work on biosynthesis of 8*R*-HPODE and (10*R*)-hydroperoxy-8*E*,12*Z*-octadecadienoic acid (10*R*-HPODE) by Aspergilli [2], we noted that 10*R*-HPODE could not be directly identified by MS/MS/MS analysis of the carboxylate anion, whereas 8*R*-HPODE was readily detected. Our first goal was to compare the stability of different hydroperoxides of 18:2n-6 and 20:4n-6 during isolation in the gas phase of an ion trap mass spectrometer. We found that the stability of the carboxylate anions of hydroperoxides differed and was critically dependent on the isolation width. Our second goal was to evaluate a silica-based chiral chromatography column (Reprosil Chiral-NR) for separation of HPODE and HPETE as free acids with on-line UV and MS/MS/MS analysis. We found that the Reprosil Chiral-NR column could resolve all available enantiomers of HPODE and the *cis-trans* conjugated HPETE in the normal phase mode. The hydroperoxides are transformed by collision-induced dissociation in the mass spectrometer to the corresponding keto fatty acids [21,22], and we also report the fragmentation of some hydroperoxides and keto fatty acids.

2. Materials and methods

2.1. Materials

18:2n-6 (99%) was from Sigma–Aldrich and 18:3n-3 (99%) was from Merck. U[^{13}C]18:2n-6 (98%), 20:4n-6 (99%) and 10-KODE were from Larodan. Hydroperoxides of 20:4n-6 and 18:2n-6 were prepared by autoxidation in the presence of α-tocopherol (67%, Sigma) [17]. Hydroperoxides were also prepared by photooxidation with methylene blue in methanol [23]. The hydroperoxides were purified from fatty acids and polar products on silica (Silicar, CC-4, Mallinckrodt, or SepPak, Waters) with increasing concentrations of diethyl ether in hexane [2]. (5*S*)-Hydroperoxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid (5*S*-HPETE), (5*S*)-hydroxyeicosa-6*E*,8*Z*,11*Z*,14*Z*-tetraenoic acid (5*S*-HETE), (12*S*)-hydroperoxyeicosa-5*E*,8*Z*,10*Z*,14*Z*-tetraenoic acid (12*S*-HPETE), (11*S*)-hydroxyeicosa-5*Z*,8*Z*,12*E*,14*Z*-tetraenoic acid (11*S*-HETE), (9*S*)-hydroxyeicosa-5*Z*,7*E*,11*Z*,14*Z*-tetraenoic acid (9*S*-HETE), (8*S*)-hydroxyeicosa-5*Z*,9*E*,11*Z*,14*Z*-tetraenoic acid (8*S*-HETE) and 8*R*-HETE were from Cayman. Hydroperoxides or alcohols with known configuration (e.g., 8*R*-HPODE, (9*S*)-hydroxyoctadeca-10*E*,12*Z*-dienoic acid (9*S*-HODE), 10*R*-HPODE, (11*S*)-hydroperoxylinoleic acid (11*S*-HPODE), (11*S*)-hydroperoxylinolenic acid (11*S*-HPOTrE), (13*R*)-hydroperoxyoctadeca-9*Z*,11*E*-dienoic acid (13*R*-HPODE), (13*R*)-hydroperoxyoctadeca-9*Z*,11*E*,15*Z*-trienoic acid (13*R*-HPOTrE), (15*S*)-hydroperoxyeicosa-5*Z*,8*Z*,11*Z*,

[2] Home page (www.dr-maisch.com/maisch/content/chiral_nr.html).

13*E*-tetraenoic acid (15*S*-HPETE) were obtained by biosynthesis using soybean lipoxygenase-1 (Lipoxidase IV; Sigma), recombinant Mn-lipoxygenase [24], tomato lipoxygenase [25], and the 8*R*- and 10*R*-dioxygenase activities of *Aspergillus fumigatus* Fresen. [2]. The hydroperoxides/alcohols were purified by RP-HPLC or NP-HPLC as described [18] and hydroperoxides were reduced to alcohols by treatment with triphenylphosphine or $NaBH_4$ (Sigma-Aldrich). Reprosil Chiral-NR column (8 μm; 250 mm × 2 mm; Dr. Maisch GmbH, Ammerbuch, Germany) was purchased locally (Dalco ChromTech). 9-, 10-, 12- and 13-ketooctadecadienoic acids (KODE) and 5-,8-, 9-, 11-, 12-, and 15-ketoeicosatetraenoic acids (KETE) were prepared by Dess-Martin oxidation of the HODE and HETE, respectively [18]. The keto compounds were separated by HPLC and characterized by UV and MS/MS analysis.

2.2. HPLC-MS analysis

Analytical RP-HPLC was performed as described [18]. For preparative separation of autoxidation products, we used a 20 mm × 150 mm column (Reprosil 100 ODS-A, 5 μm; Dr. Maisch, Ammerbuch, Germany), eluted with methanol/water/acetic acid, 90/10/0.1). NP-HPLC with on-line MS/MS and MS/MS/MS analysis was performed on silica with an analytical column (Kromasil-100SI; 250 mm × 2 mm, 5 μm, 100 Å), which was eluted at 0.3–0.5 ml/min with hexane/acetic acid, 1000/0.1, containing 1.2–1.5% isopropanol, using a ConstraMetric 3200 pump. The effluent passed a photodiode array detector (5 cm path length, Surveyor PDA, ThermoFischer). The effluent was then combined in a T-junction with isopropanol/water (3/2; 0.2–0.3 ml/min) from a Surveyor MS pump and subject to electrospray ionization in an ion trap mass spectrometer (LTQ, ThermoFischer) with monitoring of negative ions. The mixing of the two solvents reduced the stability of the spray. Therefore, the number of microscans was often increased and the Gaussian algorithm for peak smoothing was employed (Xcalibur program, setting 15). The heated transfer capillary was set at 310 °C and we used the default values for the collision energy (35; arbitrary scale) and for the *Q*-value (0.25). The ion isolation width was initially set to 1.5, 2.5, 5 or 10 amu for MS/MS analysis of hydroperoxide anions (A^-) and at 1.5 amu for sequential MS/MS analysis of A^--18. As a compromise between selectivity and signal intensity we used an isolation width of 5 amu for hydroperoxides, if not otherwise stated. $PGF_{1\alpha}$ (100 ng per min) was infused for tuning, and a service engineer of ThermoFischer recalibrated the waveforms of the instrument before this study.

CP-HPLC analysis of HPODE and HPETE was performed with Reprosil Chiral-RN, which was eluted at 0.5 ml/min with hexane/acetic acid, 1000/0.1, containing 1–1.5% of an alcoholic modifier (1.2–1.5% isopropanol, 1.2% ethanol, or 1% methanol). The effluent from the chiral column was combined with isopropanol/water (3/2; 0.3 ml/min) from a Surveyor MS pump and analyzed by MS/MS as described above.

The elution of enantiomers of 12-HPETE, 15-HPETE, and 5-HPETE was determined from retention times of 12*S*-HPETE, 15*S*-HPETE and 5*S*-HPETE, respectively. The elution of enantiomers was also determined by reducing the fractions containing separated enantiomers with triphenylphosphine and CP-HPLC analysis of the alcohols. CP-HPLC analysis of 9-HODE, 8-HETE and 11-HETE was performed with a Chiralcel OB-H column (250 mm × 4.6 mm; Daicel), which was eluted with 5 or 7% isopropanol in hexane/acetic acid, 1000/0.1 (0.5 ml/min), and combined in a T-junction with isopropanol/water to generate ions for MS/MS analysis. Steric analysis of 9-HETE was performed by analysis on Reprosil Chiral-NR with 1.2% isopropanol in hexane. Steric analysis of 5-HETE was performed on Chiralcel OC (250 mm × 4.6 mm; Daicel; eluted with 7% isopropanol in hexane at 0.5 ml/min).

3. Results

3.1. Isolation width and signal intensities of hydroperoxides and alcohols

3.1.1. HPOTrE and HPODE

We infused 13*R*-HPOTrE for electrospray ionization and measured the signal intensity of the carboxylate anion at *m*/*z* 309 in the full scan mode. As we reduced the isolation width, the signal intensity declined. The intensity was $\sim 7 \times 10^5$ using an isolation width of 5 or 7 amu, $\sim 3 \times 10^5$ at an isolation width of 2.5 amu, and reduced to $\sim 4 \times 10^3$ at 1.5 amu (thus less than 0.5% of the signal intensity at 5 and 7 amu).

We next investigated whether hydroperoxides of 18:2n-6 differed in their isolation stability during isolation and MS/MS/MS analysis in the ion trap. We first compared two non-conjugated hydroperoxides, 8*R*-HPODE and 10*R*-HPODE, formed by *A. fumigatus* [2]. 8-HPODE appeared to be more stable than 10-HPODE (Fig. 2A). We also compared a bisallylic hydroperoxide, 11*S*-HPOTrE, with a *cis-trans* conjugated hydroperoxide, 13*R*-HPOTrE. Both hydroperoxides are formed by Mn-lipoxygenase [12]. As illustrated in Fig. 2B, 11-HPOTrE was clearly detected even at an isolation window of 1.5 amu, whereas 13*R*-HPOTrE only was detected at a larger isolation width. Finally, we compared the signal intensities of the two *cis-trans* conjugated hydroperoxides, 9-hydroperoxyoctadeca-10*E*,12*Z*-dienoic acid (9-HPODE) and 13-HPODE with two non-conjugated, 10-HPODE and 12-hydroperoxyoctadeca-9*Z*,13*E*-dienoic acid (12-HPODE), obtained by photooxidation (Fig. 3). 12-HPODE yielded sufficiently strong signal for identification at 1.5 amu (not shown), and all four products were detected at 2.5 amu.

In conclusion, the signal intensities of HPODE and HPOTrE increased with a larger isolation width. Some non-conjugated hydroperoxides (8-HPODE, 11-HPODE, 11-HPOTrE, 12-HPODE) appeared to be more stable during isolation than 10-HPODE and *cis-trans* conjugated hydroperoxides (9- and 13-HPODE, 13-HPOTrE; Figs. 2 and 3).

3.1.2. HPETE

The signal intensities of 5*S*-HPETE, 12*S*-HPETE and 15*S*-HPETE were recorded during RP-HPLC-MS/MS/MS analysis (*m*/*z* 335 → *m*/*z* 317 → full scan) using an isolation width of 1.5, 2.5, 5 and 10 amu at *m*/*z* 335 (Table 1). The compounds were undetectable at 1.5 amu (signal below 1%). 15-HPETE and 12-HPETE were detectable at 2.5 amu, and the signal intensities was 20% lower at 5 amu compared to 10 amu. The increase in signal intensity from 5 amu vs. 10 amu was much larger for 5-HPETE than for 15- and 12-HPETE (Table 1). Increasing the isolation width to 30 amu did not further augment the signal of 5-HPETE.

Table 1
Total ion intensities during LC-MS/MS/MS analysis of 5-, 12-, and 15-HPETE with different isolation widths of the carboyxlate anions

Isolation width	Total ion current (%)[a]		
(amu)	15-HPETE	12-HPETE	5-HPETE
1.5	<1.5	<1.5	0
2.5	34	30	<1.5
5	82	81	43

[a] The area under the Gaussian curves of the total ion current during MS/MS/MS analysis (*m*/*z* 335 → *m*/*z* 317 → full scan) were measured during RP-HPLC analysis and expressed in percent of the area under the peaks obtained with an isolation width of 10 amu (set to 100%). The HPETEs were injected three times and each time analyzed with four isolation widths (1.5, 2.5, 5 and 10 amu) in otherwise identical acquisition programs.

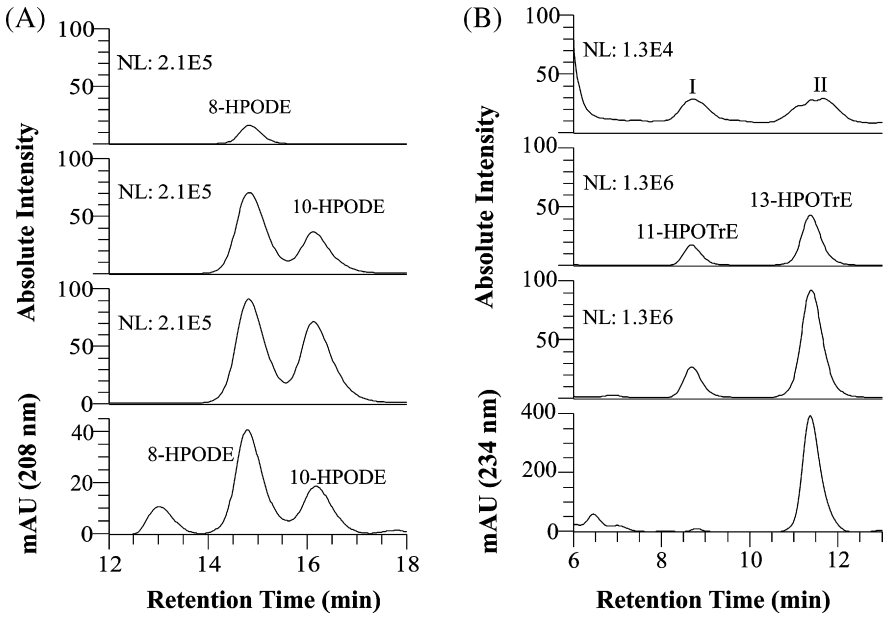

Fig. 2. LC-MS/MS analysis of 8*R*-HPODE, 10*R*-HPODE, 11*S*-HPOTrE and 13*R*-HPOTrE using three different isolation widths. (A) 8- and 10-HPODE were separated by RP-HPLC and analyzed with an isolation width of 1.5, 2.5 and 5 amu, respectively (first, second and third chromatogram). The traces show the total ion current during MS/MS/MS analysis (m/z 311 → m/z 293 (isolation width 1.5 amu) → full scan). The bottom chromatogram shows UV analysis with monitoring of 208 nm. (B) 11*S*- and 13*R*-HPOTrE were separated by RP-HPLC and analyzed by MS/MS/MS (m/z 309 → m/z 291 (isolation width 1.5 amu) → full scan. The signal intensities increased with the isolation width of 1.5, 2.5 and 5 amu, as shown by the three chromatograms, respectively. Peak I of the top chromatogram yielded a characteristic MS/MS/MS spectrum of 11-HPOTrE, whereas 13-HPOTrE/13-KOTrE could not be detected in peak II with this isolation width (1.5 amu). NL, normalized intensity (2.1×10^5 was set to 100%). Bottom, UV analysis with monitoring at 234 nm. Monitoring at 208 nm suggested that 11- and 13-HPOTrE were present in a ratio of ~1:4.

3.1.3. HOTrE

In contrast to the hydroperoxides, we found that the signal intensities of two alcohols, 11- and 13-HOTrE, did not increase significantly during MS/MS by raising the isolation width (from 1.5 to 2.5 and 5 amu).

3.2. Enantiomeric separation of hydroperoxides of 18:2n-6 and 20:4n-6

3.2.1. Analysis of HPODE with isopropanol as alcoholic modifier

The separation of enantiomers of 9-, 10-, 12- and 13-HPODE on Reprosil Chiral-NR is shown in Fig. 4A. The elution order of the *cis-trans* conjugated hydroperoxides was 13*S*-HPODE, followed by 9*S*-HPODE and 13*R*-HPODE (partly resolved) and 9*R*-HPODE using 1.5% isopropanol as alcoholic modifier. The corresponding alcohols eluted before the corresponding hydroperoxides. The enantiomers of 10-HPODE were resolved (10*S*-HPODE eluted before 10*R*-HPODE) as well as the two enantiomers of 12-HPODE (unknown elution order). These compounds could be detected by reconstructed ion chromatograms using characteristic ions (Table 2).

We also examined 11-HPODE and 8-HPODE. Rac 11-HPODE was partly separated on the Reprosil Chiral-NR column into the 11*R* and 11*S* stereoisomers as shown in Fig. 5A using 1.2% isopropanol as modifier. The 11*R* enantiomer eluted before the 11*S* enantiomer.

8-HPODE from biosynthesis (*A. fumigatus*) and autoxidation was purified by RP- and NP-HPLC. Rac 8-HPODE was resolved as shown in Fig. 5B, and the *S* stereoisomer eluted before the *R* stereoisomer (resolution ~1.1). Analysis of the biologically produced 8-HPODE by *A. fumigatus* yielded 3% of the *S* and 97% of the *R* stereoisomers in agreement with previous results [18].

3.2.2. Analysis of HPODE with methanol as alcoholic modifier

The separation of the regioisomeric *cis-trans* conjugated HPODE was enhanced using 1% methanol as alcoholic modifier compared to 1.2% isopropanol, as indicated in Fig. 4B. However, the resolution of the pairs of enantiomers of 9-, 10-, 12, and 13-HPODE was not improved.

Ethanol (1.2%) as alcoholic modifier did not alter the resolution obtained with 1.2% isopropanol.

3.2.3. Analysis of HPETE with isopropanol or methanol as alcoholic modifiers

Reprosil Chiral-NR separated the six *cis-trans* conjugated HPETEs, which were obtained by autoxidation, with isopropanol (1.2%) and with methanol (1%) as alcoholic modifiers as shown in Figs. 6 and 7. A comparison of 1.2% isopropanol with 1% methanol as alcoholic modifiers showed that isopropanol enhanced the reso-

Table 2
Characteristic and intense ions formed during LC-MS/MS/MS analysis of carboxylate anions of HPODE and HPETE

HPODE	→KODE	Characteristic ions (m/z)[a]				Reference
8-HPODE	→8-KODE	171	183			[21]
9-HPODE	→9-KODE	185	197			[20,25]
10-HPODE	→10-KODE	139	153			
11-HPODE	→11-KODE	151	177			[21]
12-HPODE	→12-KODE	165	191			
13-HPODE	→13-KODE	113	179	195		[20,25]
HPETE	**→KETE**	**Characteristic ions (m/z)[a]**				**Reference**
5-HPETE	→5-KETE	129	203	163		[20]
8-HPETE	→8-KETE	111	163	169	205	
9-HPETE	→9-KETE	151	193	219		
11-HPETE	→11-KETE	123	149	165		
12-HPETE	→12-KETE	153	161	179		[20]
14-HPETE	→14-KETE	125	165	191		
15-HPETE	→15-KETE	113	219	203		[20]

[a] MS/MS/MS analysis (A^- → (A^--18) → full scan) also yielded intense signals and base-peaks at A^--(18 + 44), due to sequential loss of water and CO_2, and other ions, as illustrated in Figs. 8 and 9. The MS/MS/MS spectra of the hydroperoxides appear to be identical to the corresponding keto compounds.

Table 3
Resolution of enantiomers of HPETEs as free acids on Reprosil Chiral-NR

	Resolution	
	Hexane/isopropanol (100/1.2)	Hexane/methanol (100/1)
5-HPETE	1.1	0.9
8-HPETE	1.6	1.1
9-HPETE	ND	1.2
11-HPETE	1.5	1.6
12-HPETE	1.7	1.2
15-HPETE	2.1	1.4

The *S* enantiomer eluted before the *R* entantiomer with exception of 8-HPETE. Enantiomers of 9-HPETE could not be identified in the complex mixture of hydroperoxides, which were only partly resolved with 1.2% isopropanol as alcoholic modifier. ND, not determined.

lution of enantiomers, whereas methanol improved the resolution of regioisomeric hydroperoxides (Table 3; Figs. 6 and 7).

The *cis-trans* conjugated enantiomers with *S* configuration eluted before the enantiomers with *R* configuration with one exception, 8-HPETE. With 1% methanol as modifier, the regioisomeric HPETEs were partly resolved, except 11-HPETE and 15-HPETE. The *S* enantiomers of 11- and 15-HPETE co-eluted and so did the *R* enantiomers of 11- and 15-HPETE, as shown in Fig. 7. 9-HPETE and 5-HPETE yielded strong UV signals, but the intensity of the total ion current of 9-HPETE and 5-HPETE was much lower than for the other HPETEs at an isolation width of 5 amu. Increasing the isolation width to 10 amu more than doubled the signal of 5-HPETE, as discussed above.

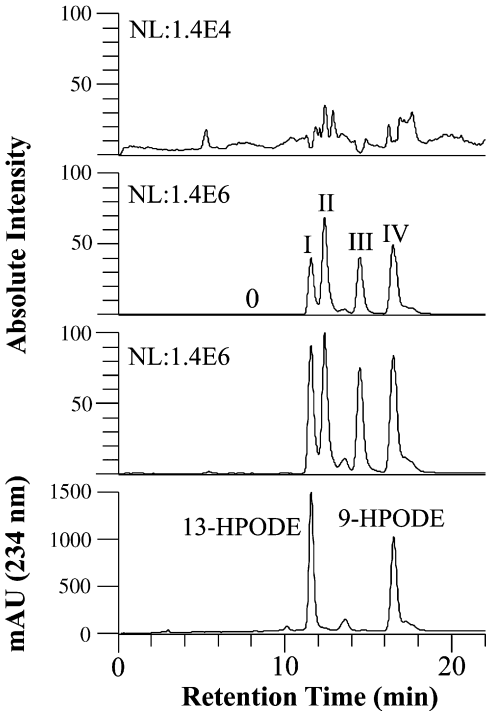

Fig. 3. LC-MS/MS analysis of hydroperoxides of 18:2n-6 obtained by photooxidation at different isolation widths of the hydroperoxide anions. Top, the trace shows the total ion current in the MS/MS/MS analysis: *m/z* 311 (isolation width 1.5 amu) → *m/z* 293 (isolation width 1.5 amu) → full scan. The hydroperoxides were not detected except for traces of 12-HPODE. The second trace shows the total ion current in the MS/MS/MS analysis *m/z* 311 (isolation width 2.5 amu) → *m/z* 293 (isolation width 1.5 amu) → full scan, and all four HPODE were detected, and 12-HPODE (peak II) yielded the strongest signal. Peak I, III and IV contained 13-, 10-, and 9-HPODE. The third trace shows the total ion current in the MS/MS/MS analysis *m/z* 311 (isolation width 5 amu) yielding a further improved signal intensity. NL, normalized intensity (1.3×10^4 set to 100% at top, and 1.3×10^6 set to 100% in second and third trace). The bottom trace shows UV analysis with monitoring at 234 nm.

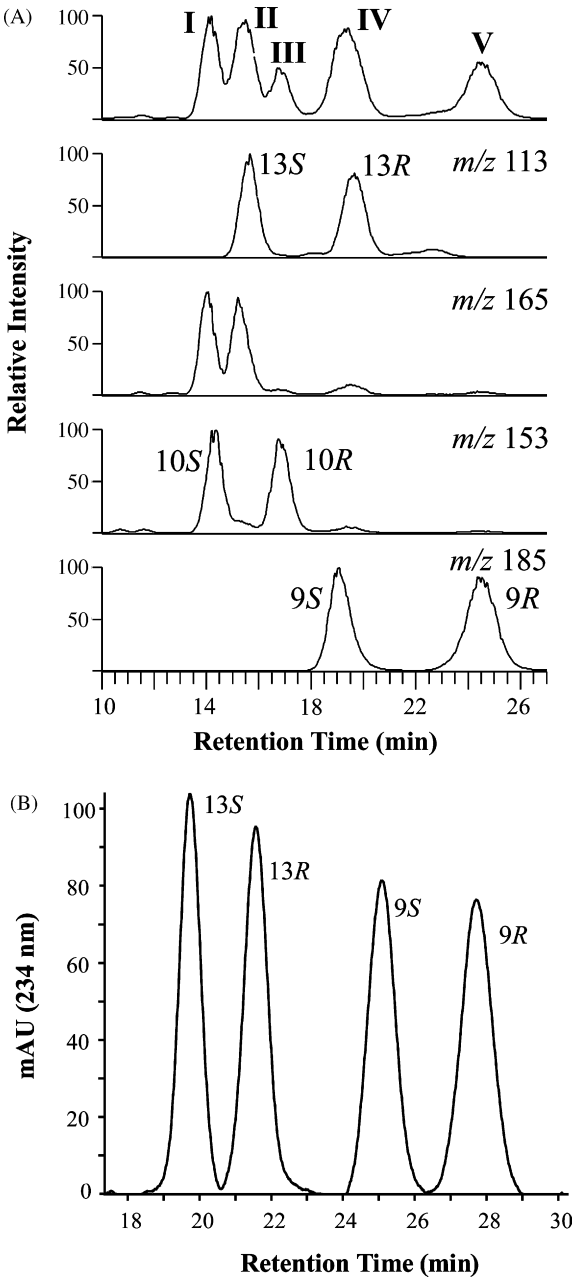

Fig. 4. Separation of HPODE on Reprosil Chiral-NR with isopropanol and with methanol as modifier and with detection of characteristic ions. (A) LC-MS analysis with isopropanol as modifier. Top, Total ion current showed five distinct peaks. The reconstructed ion chromatograms below were used to identify the enantiomers: *m/z* 113 (13-HPODE), *m/z* 165 (12-HPODE), *m/z* 153 (10-HPODE) and *m/z* 185 (9-HPODE). (B) Separation of enantiomers of 13- and 9-HPODE with 1% methanol as modifier (UV analysis), as indicated by the lettering.

Photooxidation of 20:4n-6 yielded a similar profile of *cis-trans* conjugated hydroperoxides. In addition, 14-hydroperoxyeicosa-5*Z*,8*Z*,11*Z*,15*E*-tetraenoic acid was tentatively identified as two partly separated enantiomers (1.2% isopropanol), which eluted between the right shoulder of 12*S*-HPETE and 12*R*-HPETE, and showed expected fragments during MS/MS/MS analysis.

3.3. Enantiomeric separation of HETE and HODE

3.3.1. Reprosil Chiral-NR

We found that the enantiomers of 9-HETE were resolved with 1.2% isopropanol as alcoholic modifier, but the other HETEs were

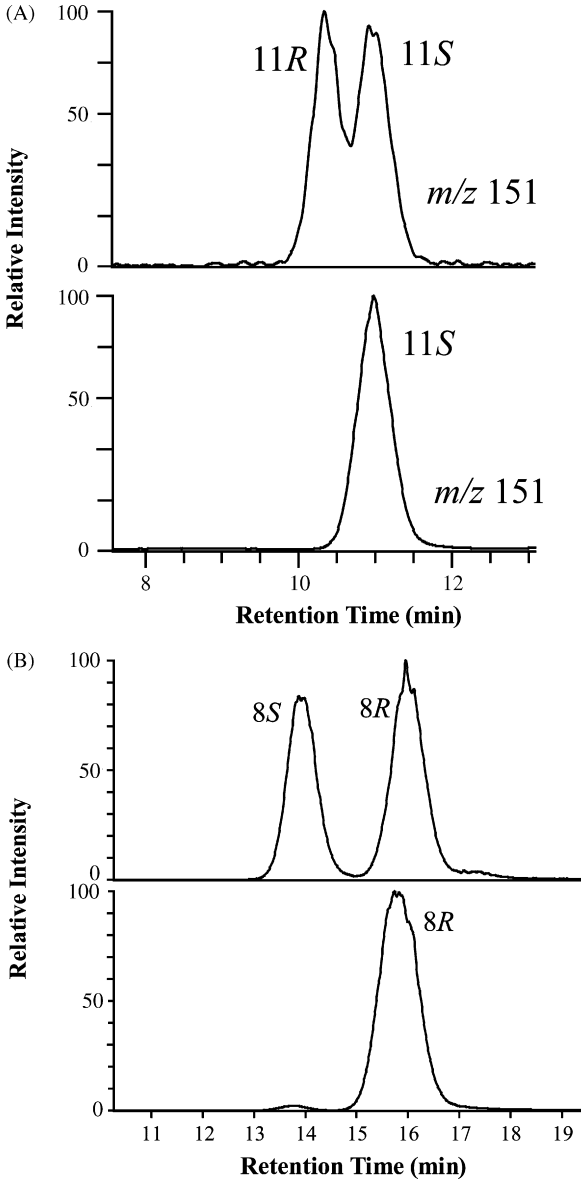

Fig. 5. Chromatography of 11-HPODE and 8-HPODE on Reprosil Chiral-NR. (A) Partial separation of the enantiomers of 11-HPODE on Reprosil Chiral-NR. Top, analysis of rac 11-HPODE. Bottom, analysis of 11*S*-HPODE. The figure shows reconstructed ion chromatograms of MS/MS/MS analysis (*m/z* 151). (B) Separation of rac 8-HPODE (top) and 8*R*-HPODE (bottom). The figure shows reconstructed ion chromatograms of MS/MS/MS analysis (*m/z* 171).

not separated. The elution order was the same as for 9-HPETE (9-HPETE eluted before 9*R*-HPETE). The enantiomers of 10-HODE can be resolved on Reprosil Chiral-NR [18]. The enantiomers of 12-HODE were also partly resolved.

3.3.2. Chiralcel OB, OC, and OD

The six *cis-trans* conjugated HETEs and 9-HODE were separated as free acids as follows: 15-HETE, 12-HETE, 11-HETE, 8-HETE and 9-HODE on Chiralcel OB-H [7,11]; 5-HETE on Chiralcel OC (5*R*-HETE eluted before 5*S*-HETE) with 5–7% isopropanol; 15-HETE on Chiralcel OD-H (10% isopropanol)[3].

[3] Unpublished observations.

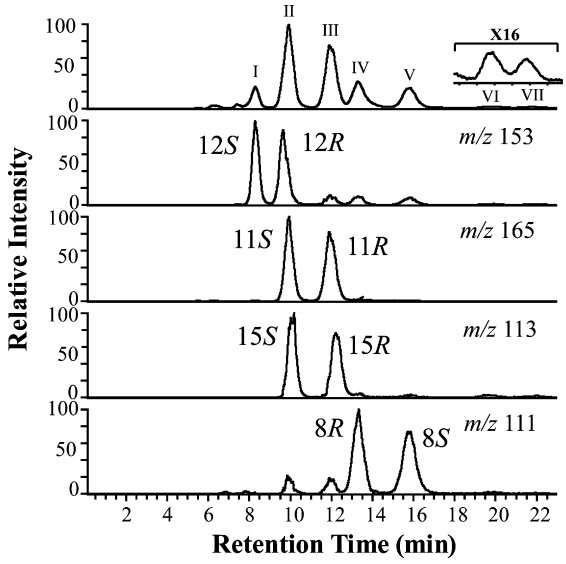

Fig. 6. Separation of HPETEs on Reprosil Chiral-NR with isopropanol as alcoholic modifier. LC-MS/MS/MS analysis (*m/z* 335 → *m/z* 317 → full scan) showing the total ion current and selective ion monitoring of characteristic ions of 12-HPETE, 11-HPETE, 15-HPETE, and 8-HPETE, respectively.

3.4. MS/MS/MS analysis

3.4.1. HPODE and KODE

HPODEs were converted to keto compounds in the mass spectrometer [21,26], but the fragmentation of 10- and 12-KODE has not been investigated. We therefore prepared U[^{13}C]12-HPODE and U[^{13}C]10-HPODE for comparison with 12- and 10-KODE.

The MS/MS/MS spectrum of 12-HPODE (*m/z* 311 → *m/z* 293 → full scan) was almost identical to the MS/MS spectrum of 12-KODE (*m/z* 293 → full scan). The MS/MS/MS spectrum of U[^{13}C]12-HPODE and the MS/MS spectrum of 12-KODE are shown in Fig. 8. The MS/MS spectrum of 12-KODE (*m/z* 293 → full scan) showed two strong signals at *m/z* 191 and *m/z* 165, in addition to fragments formed by loss of water (*m/z* 275) and CO_2 (*m/z* 249). The corresponding ions of the MS/MS/MS spectrum of U[^{13}C]12-HPODE (*m/z* 329 → *m/z* 311 → full scan) were apparently present

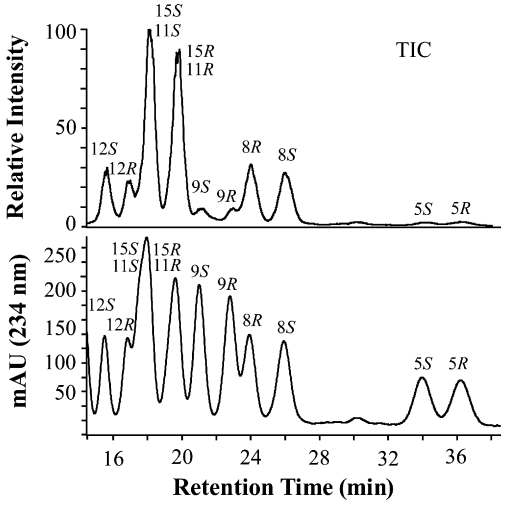

Fig. 7. Separation of HPETEs on Reprosil Chiral-NR with methanol as alcoholic modifier. Top, total ion current from MS/MS/MS analysis (*m/z* 335 → *m/z* 317 → full scan). The ion intensities of 9-HETE and 5-HETE were low compared to the other HPETEs. Bottom, UV analysis (234 nm). The enantiomers eluted as indicated. The *S* and *R* enantiomers of 15-HETE and 11-HETE co-eluted. TIC, total ion current.

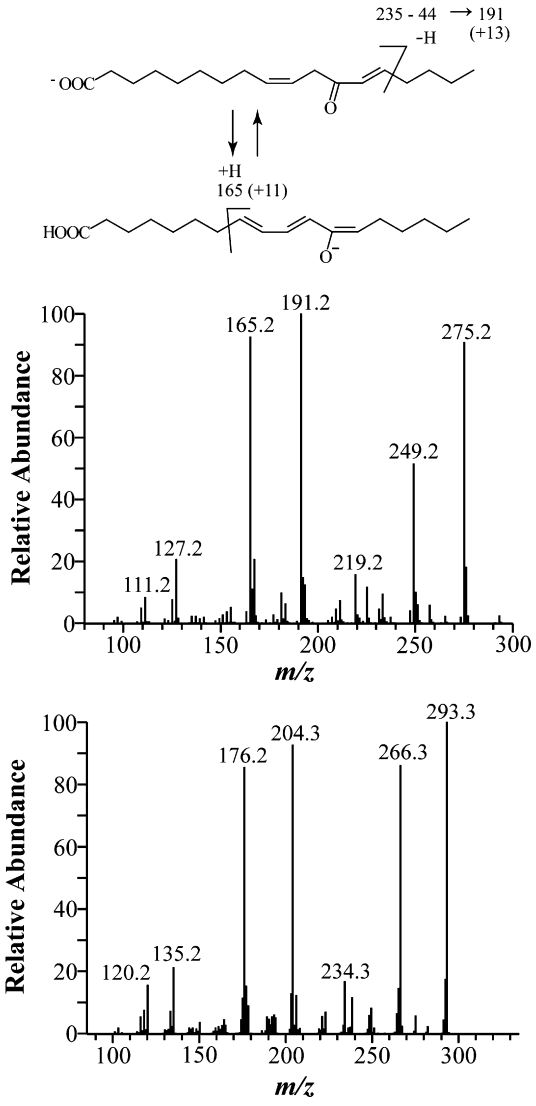

Fig. 8. MS analysis of 12-KODE and U [^{13}C]12-HPODE. 12-KODE was analyzed by MS/MS analysis of the carboxylate anion (*m/z* 293, top) and U[^{13}C]12-HPODE by MS/MS/MS analysis of the carboxylate anion (*m/z* 327 → *m/z* 311 → full scan). The fragments *m/z* 165 and *m/z* 191 might possibly be formed in the gas phase of the ion trap, as suggested by the chemical structures on top.

at *m/z* 204 (191 + 13) and *m/z* 176 (165 + 11). The intense signals at *m/z* 191 and *m/z* 165 were further studied by MS/MS/MS analysis of 12-KODE. Analysis of the daughter ion formed by loss of water (A^--18; *m/z* 293 → *m/z* 275 → full scan) yielded signals both at *m/z* 191 and *m/z* 165, whereas analysis of the daughter ion formed by loss of CO_2 (A^--44; *m/z* 293 → *m/z* 249 → full scan) only yielded the latter. Analysis of the fragmentation of the ion at *m/z* 191 (*m/z* 293 → *m/z* 191 → full scan) yielded a base-peak at *m/z* 173 (191 − 18), whereas analysis of *m/z* 165 (*m/z* 293 → *m/z* 165 → full scan) yielded signals at both *m/z* 147 (165 − 18) and *m/z* 111 (165 − 44). This suggested that the signal at *m/z* 191 was formed by loss of CO_2 and C_4H_{10}. The signal at *m/z* 165 was possibly due to cleavage between C-7 and C-8, as suggested in Fig. 8.

A comparison of the MS/MS spectrum of 10-KODE and the MS/MS/MS/spectrum of U[^{13}C]10-HPODE suggested that the signal at *m/z* 153 in the spectrum of 10-KODE was shifted to *m/z* 163 (153 + 10) in the MS/MS/MS spectrum of U[^{13}C]10-HPODE, suggesting formation of a fragment with 10 carbons (possibly $H_2C{=}C(O^-){-}CH_2{-}CH{=}CH{-}C_5H_{11}$).

3.4.2. HPETE and KETE

The MS/MS/MS spectra of 5-, 12-, and 15-HPETE were identical with the MS/MS spectra of 5-, 12-, and 15-KETE, as reported by MacMillan and Murphy [21]. The MS/MS spectra of 8-KETE, 9-KETE and 11-KETE were not reported, and they are shown in Fig. 9. Characteristic ions are summarized in Table 2. The fragmentation mechanisms proposed by the inserts in Fig. 9 was supported by MS/MS/MS analysis (*m/z* 317 → *m/z* 299 → full scan; *m/z* 317 → *m/z* 273 → full scan).

Due to the shift in position of the keto groups in 5-KETE, 8-KETE and 11-KETE, the signals at *m/z* 203 and *m/z* 129 of 5-KETE could be related to the signals at *m/z* 163 (203 − 40) and *m/z* 169 (129 + 40, weak) of 8-KETE (Table 2). In analogy 15-KETE, 12-KETE and 9-KETE showed signals at *m/z* 113, *m/z* 153 (113 + 40) and *m/z* 193 (113 + 80), respectively, possibly due to fragments carrying the ω end. 15-KETE and 12-KETE also showed signals at *m/z* 219 and *m/z* 179 (219 − 40), respectively. Fragment ions can be formed by many mechanisms, and some are not specific for the position of the keto groups (*cf.* Table 2). The MS/MS/MS spectrum of 14-HPETE showed signals, *inter alia*, at *m/z* 191, *m/z* 165 (likely $H_2C{=}CH{-}CH{=}CH{-}C(O^-){=}CH{-}C_5H_{11}$, in analogy with this fragment of 12-HPODE discussed above) and *m/z* 125 ($H_2C{=}CH{-}C(O^-){=}CH{-}C_5H_{11}$).

3.5. Biological application

Mn-lipoxygenase oxidizes [^{14}C]18:2n-6 to 11*S*-HPODE (∼17%), 13*R*-HPODE (∼80%) and a few percent 9-HPODE [27,28]. The products formed by recombinant Mn-lipoxygenase were analyzed by CP-HPLC-MS/MS. The total ion current showed 11*S*- and 13*R*-HPODE as major products, whereas 9-HPODE was detected by selective ion monitoring (Fig. 10). The major isomer of 9-HPODE had the same retention time as 9*S*-HPODE, and the 9*S* and 9*R* stereoisomers were formed in a 6:1 ratio.

3.6. Regeneration

Operated in RP mode (methanol/water/acetic acid, 65/35/0.01), the Reprosil Chiral-NR column did not separate the enantiomers of HPETE. The Reprosil Chiral-NR column was successfully regenerated for normal phase chromatography by washing (0.3 ml per min) with isopropanol, ∼30 column volumes of hexane/dimethoxypropane/acetic acid, 95/2.5/2.5, and 2% isopropanol in hexane, as described for regeneration of silica columns [29].

4. Discussion

We report that Reprosil Chiral-NR is useful for separation of HPODE and HPETE as free acids, in many cases with base-line resolution. Resolution of enantiomers was improved with isopropanol as alcoholic modifier compared to methanol, but separation of the regioisomeric hydroperoxides appeared to be superior with methanol. This column provides an alternative to the more expensive but versatile columns of the Chiralcel and Chiralpak series[1] [15,17].

Many biological studies on oxygenation of fatty acids are complicated due to reduction of hydroperoxides by enzymatic or non-enzymatic routes to alcohols. It was therefore disappointing that the Reprosil Chiral-NR column only separated the enantiomers of 10- and 12-HODE and 9-HETE. Nevertheless, this column appeared to have a large capacity for enantiomeric separation of different hydroperoxy fatty acids as free acids. The *S* stereoisomers eluted before the *R* stereoisomers with exception of 8-HPETE and 11-HPODE. The elution order of hydroperoxide enantiomers must therefore be confirmed by analysis of stereoisomers with known

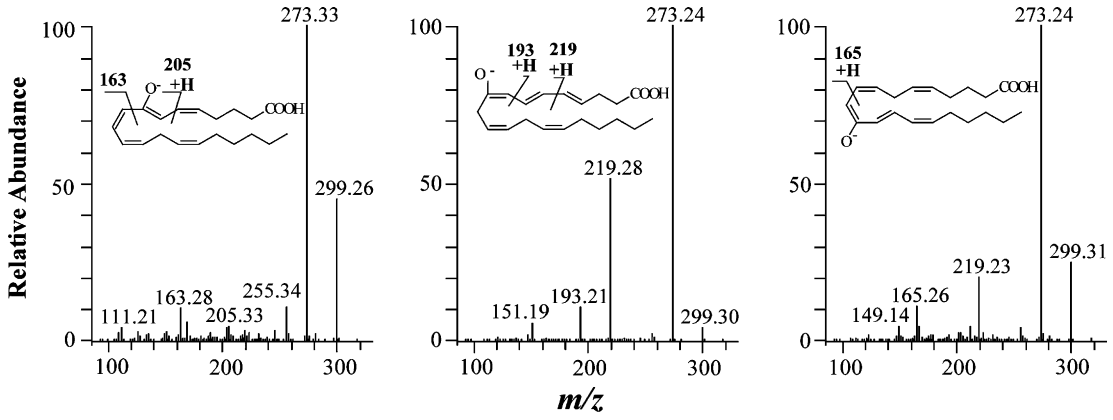

Fig. 9. MS/MS spectra of 8-KETE, 9-KETE and 11-KETE. The inserts suggest cleavage points for generating of characteristic ions, as indicated. The keto compounds could be subject to keto-enol tautomerism, as indicated by the insets.

configuration, or confirmed by steric analysis of the corresponding alcohols after reduction.

The separation of hydroperoxids is of practical value for several reasons. The profile of hydroperoxides formed by lipoxygenases and other fatty acid dioxygenases are often used for classification and as a direct proof of a dioxygenase reaction. This can be illustrated by analysis of products formed by Mn-lipoxygenase. The latter forms 11*S*-HPODE, 13*R*-HPODE and small amounts of 9-HPODE [27,28]. Steric analysis of 9-HPODE by CP-HPLC-MS/MS showed that 9*S*-HPODE was the major stereoisomer. Binding in the opposite direction ("head first") would generate 9*R*-HPODE. This suggests that 18:2n-6 is strictly bound with the ω-end in the active site ("tail first") so that oxygen is inserted at C-13, C-11 or C-9 at one side of the pentadienyl radical, as illustrated in Fig. 10. For comparison, soybean lipoxygenase can bind 18:2n-6 both "head first" and "tail first" in a pH dependent manner [30].

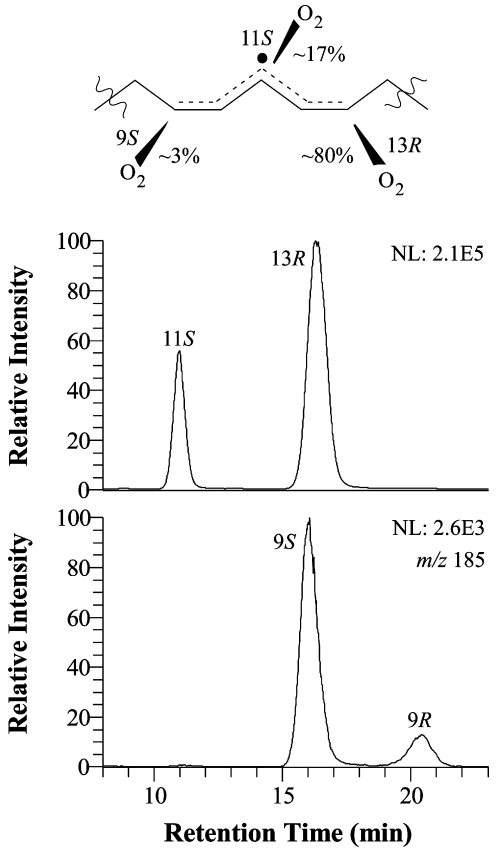

Fig. 10. Stereospecific oxygenation of 18:2n-6 by Mn-lipoxygenase. The hydroperoxides were separated by CP-HPLC (Reprosil Chiral-NR) using 1.2% isopropanol as alcoholic modifier. The top chromatogram shows the total ion current (TIC) of MS/MS analysis (*m/z* 311 → *m/z* 293 → full scan) and the two major products formed by oxidation at C-13 and C-11. The bottom chromatogram shows selective reaction monitoring of *m/z* 185, a characteristic fragment of 9-HPODE (Table 2). 9*S*- and 9*R*-HPODE were formed in a ratio of ~6:1, and constituted only a few percent of the total products. NL, normalized intensity (2.1×10^5 was set to 100% in the top chromatogram and 2.6×10^3 to 100% in the bottom chromatogram). The top drawing illustrates the stereospecific oxygenation at C-13, C-11, or C-9 of the pentadienyl radical formed by Mn-lipoxygenase, and shows the relative amounts of the three metabolites in percent.

Hydroperoxides can be converted to biologically active compounds, and it is therefore a need to isolate them for further studies. To this respect, hydroperoxides can be obtained by biosynthesis or by autoxidation or photooxidation of polyunsaturated fatty acids [17]. 12*S*-HPETE, 8*S*-HPETE, and the enantiomers of 5-HPETE were resolved on Reprosil Chiral-NR from other HPETEs formed during autoxidation of 20:4n-6. 12*S*-HPETE eluted first, but the elution order of 12*S*-and 12*R*-HPETE can be reversed on a column, which contains the opposite enantiomeric chiral selector[2]. It should therefore be possible to purify 12*R*-HPETE from autoxidation on the alternative column as the first eluting hydroperoxide. In comparison, several steps of chromatography will be needed to purify the enantiomers of 8-HPODE from autoxidation of 18:2n-6 for biological studies, as 8-HPODE is formed only as a minor product, about 1% [31]. The 8*R* enantiomer is of interest as the precursor of the hydroperoxide isomerase activity of 5,8-, 7,8-, and 8,11-linoleate diol synthases [2].

The hydroperoxides were assayed on-line by UV analysis and electrospray ionization with monitoring of negative ions during MS/MS/MS analysis. The hydroperoxides are transformed in the mass spectrometer to the corresponding keto fatty acids [21,22]. Unfortunately, the fragmentation of keto fatty acids is complicated by keto-enol tautomerism [26]. We therefore prepared KETE, KODE and U[^{13}C]HPODE as references. This confirmed that the MS/MS/MS spectra of 8-, 9-, and 11-HPETE were virtually identical to the MS/MS spectra of 8-, 9-, and 11-KETE, as previously reported for 5-, 12- and 15-HPETE and 5-, 12- and 15-KETE [21]. The fragmentation of 12-HPODE was further complicated by migration of the double bonds during keto-enol tautomerism, as illustrated by the fragmentation of 12-HPODE and U[^{13}C]12-HPODE (Fig. 8). We found that the chemical stability of the hydroperoxides varied several orders of magnitude with the isolation width (1.5–10 amu) during MS/MS analysis in an ion trap. The signals of 11- and 13-HOTrE were stable during isolation and MS/MS analysis, whereas the signals of the corresponding hydroperoxides increased with the isola-

tion width. 5-HPETE and other *cis-trans* conjugated hydroperoxides appeared to be least stable, whereas non-conjugated hydroperoxides (8-HPODE, 11-HPODE, 11-HPOTrE and 12-HPODE) often yielded sufficiently strong signals even at an isolation width of 1.5 or 2.5 amu.

Why are hydroperoxides unstable during isolation in the ion trap? Ions are trapped by a combination of radial and axial motion created by the applied radio frequency and voltage on the four hyperbolic rods of the trap [32]. The isolation width is created by changing the electrical fields so that all ions above and below a certain m/z range are ejected. A very narrow window increases the energy of the selected ions, and may cause brake-down of unstable compounds.

In summary, we have shown that enantiomers of hydroperoxides of 18:2n-6 and 20:4n-6 can be separated as free acids by CP-HPLC (Reprosil Chiral-NR) with resolution of many regioisomers. MS/MS analysis of hydroperoxides in an ion trap should be performed using a relatively large isolation width, and this may also apply to analysis of other unstable compounds.

Acknowledgements

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