Separation and Identification of Phospholipid Peroxidation Products

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ABSTRACT: The molecular species in mixtures of phospholipid hydroperoxides are difficult to separate and identify by typical chromatographic and mass spectrometric techniques. As reported by Havrilla and coworkers, silver ion coordination ion-spray mass spectrometry (CIS–MS) has proven to be a powerful technique for the identification of mixtures of hydroperoxides. This ionization technique, which involves the formation of Ag^+ adducts of the hydroperoxides, provides valuable, unambiguous structural information about the hydroperoxides. Herein, we report a method for the analysis and identification of phospholipid hydroperoxides using CIS–MS. We also report an improved method for the separation of phospholipid hydroperoxides by reversed-phase high-performance liquid chromatography (RP–HPLC), which, for the first time, separates some of the hydroperoxide isomers. CIS–MS can be coupled with this RP–HPLC method by the addition of $AgBF_4$ to the mobile phase or to the HPLC effluent postcolumn, thus allowing powerful HPLC–MS techniques to be used to identify complex mixtures of phospholipid hydroperoxides.

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Lipid peroxidation has been widely studied because of its implicit role in the pathogenesis of a number of human diseases including cancer (1), neurodegenerative diseases (2,3), and atherosclerosis (4). Considerable evidence supports the hypothesis that oxidative modification of the lipids in low density lipoproteins (LDL) may play an important role in the onset of atherosclerosis (5). Phospholipids, as the major lipid components of the surface layer of LDL, are a primary target for oxidation (6). Linoleic acid and arachidonic acid are the most abundant unsaturated fatty acids esterified to phospholipids in LDL. Lipids containing these fatty acids are especially susceptible to free radical chain oxidation (7–9).

Some two decades ago, we reported that neat films of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine (PLPC) and 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine (SAPC) react readily with oxygen at room temperature to give a mixture of phospholipid hydroperoxides (10). These oxidized products could be separated from the unoxidized phospholipid using reversed-phase high-pressure liquid chromatography (RP–HPLC). However, separation of the molecular species of the oxidized phospholipid could not be achieved with the chromatographic options available at that time. Since then, improvements have been made in the HPLC separation of intact phospholipid molecular species. The addition of ion-pairing agents, such as ammonium acetate (11) and choline chloride (12), to HPLC mobile phases leads to improved separation and peak shape.

These improvements in chromatography along with advances in mass spectrometry (MS) have helped make the analysis of intact phospholipid hydroperoxides possible. In 1994, Zhang *et al.* (13) reported the first method to analyze intact hydroxyeicosatetraenoyl-*sn*-glycero-3-phosphatidylcholine (HETE PC) species by fast atom bombardment–tandem mass spectrometry (FAB–MS/MS). Subsequently, an HPLC–MS technique (thermospray MS) used to analyze phospholipid hydroperoxides has been reported (14), and electrospray MS (ESI–MS) has been applied to phospholipid hydroperoxide analysis (15–17).

Analyzing phospholipid hydroperoxides by positive ion ESI–MS allows detection of the parent ion, but MS/MS experiments do not give structural information about the position of oxidation on the side chain. Only the mass-to-charge ratio (*m/z*) of the phosphocholine head group can be detected because it is the only part of the molecule that is charged. The negative ion ESI–MS/MS experiments reported by Hall and Murphy (16) do give structural information about the position of oxidation on the side chains when a high orifice potential is applied during the ionization process. The high potential causes in-source fragmentation of the phospholipid species so that the parent ion, as well as two daughter ions that correspond to the *m/z* for the carboxylate anions of the fatty acid side chains, is observed. Collision-induced dissociation (CID) experiments where the ion with the *m/z* for the oxidized fatty acid of interest is activated give fragment ion peaks that are indicative of the position of oxidation on the side chain. These studies only report separation of the

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Abbreviations: BHT, butylated hydroxytoluene; CD, circular dichroism; CE, Cotton effect; CID, collision-induced dissociation; CIS–MS, coordination ion-spray mass spectrometry; DLI, direct liquid infusion; ESI–MS, electrospray MS; FAB–MS, fast atom bombardment MS; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high-pressure liquid chromatography; HPODE, hydroperoxyoctadecadienoic acid; LC, liquid chromatography; LDL, low density lipoproteins; MS, mass spectrometry; methyl-9-HETE, methyl 9-hydroxy-5(*Z*),8(*E*),11(*Z*),14(*Z*)-eicosatetraenoate; MS/MS, tandem MS; NMR, nuclear magnetic resonance; ODS, octadecylsilane; PC, phosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidycholine; PLPC-OH, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidycholine alcohol; PLPC-OOH, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidycholine hydroperoxide; PMC, pentamethylchromanol; PPh_3, triphenylphosphine; RP, reversed phase; SAPC, 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine; SRM, selected reaction monitoring; TLC, thin-layer chromatography; UV, ultraviolet.

phospholipid species by class according to their head group and not by the position of oxidation on the side chains.

As an alternative to protonation, organic molecules containing hard or soft Lewis basic sites can be ionized by cations such as Li^+, Na^+, or Ag^+ (18–20). This ionization technique involves the formation of charged analyte complexes formed by the addition of a suitable coordination ion to the analyte. Combined with ESI–MS, coordination ion-spray mass spectrometry (CIS–MS) (21) has proved useful for analyzing complex mixtures of cholesteryl ester peroxides (22). Herein we report a method for the analysis and identification of phospholipid hydroperoxides using CIS–MS that eliminates some of the problems associated with ESI–MS. We also report an improved HPLC method that separates some of the isomers of linoleate hydroperoxides derived from PLPC and all of the arachidonate hydroperoxide isomers derived from SAPC.

MATERIALS AND METHODS

Phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL) or from Sigma Chemical Company (St. Louis, MO) and used without further purification. PLPC was purchased as a powder and SAPC was purchased as a chloroform solution. Solvents, such as methanol, water, and 2-propanol, were HPLC quality and purchased from either Fisher Chemical (Phillipsburg, NJ) or EM Science (Gibbstown, NJ). Hexanes were purchased from Burdick & Jackson (Muskegon, MI). All other reagents were purchased from Aldrich Chemical Company (Milwaukee, WI) and used without further purification.

Reactions involving hydroperoxides were monitored by thin-layer chromatography (TLC) using a stain of 1.5 g of *N,N′*-dimethyl-*p*-phenylenediamine dihydrochloride/25 mL of H_2O/125 mL of MeOH/1 mL acetic acid. Hydroperoxides yield an immediate pink color. TLC was carried out using 0.2-mm layer thickness, silica-coated aluminum columns (EM Science) that were visualized at 254 nm, phosphomolybdic acid char, or the peroxide stain. In general, hydroperoxides were stored as dilute solutions with 1 mol% butylated hydroxytoluene (BHT) in benzene at −78°C and never exposed to temperatures >40°C.

Instruments. Analytical HPLC was conducted on a Waters model 600 HPLC instrument, with a Waters model 486 tunable absorbance detector operating at 234 nm and with output to a Hewlett-Packard 3396 Series III integrator. Semipreparative HPLC was conducted on a Waters model 600E HPLC instrument with a Waters model 481 variable wavelength detector operating at 234 nm and with output to a Fisher Record-All Series 5000 strip chart recorder. Chiral HPLC was conducted on a Waters model 590 instrument with a Hewlett-Packard 1040A photodiode array detector. Semipreparative chiral HPLC was conducted on a Waters model 600 instrument with an LDC/Milton Roy Spectromonitor 3000 variable wavelength detector operating at 234 nm and with output to a Hitachi D-2500 Chromato-Integrator.

^{1}H Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker DRX-400 (400 MHz) spectrometer in $CDCl_3$. Immediately prior to sample preparation, $CDCl_3$ was passed through a plug of basic alumina to remove any HCl.

Mass spectrometry. CIS–MS was performed using a Finnigan Thermoquest TSQ-7000 (San Jose, CA) triple quadrupole mass spectrometer equipped with a standard API-1 electrospray ionization source outfitted with a 100-µm deactivated fused-Si capillary. Data acquisition and spectral analysis were conducted using ICIS software, version 8.3.2, running on a Digital Equipment Alpha Station 200 4/166. Nitrogen gas served both as the sheath and auxiliary gas; argon served as the collision gas. The electrospray needle was maintained at 4.6 kV, and the heated capillary temperature was 250°C. The tube lens and capillary voltages were optimized to maximize ion current for electrospray; the optimal values were determined to be 90 and 10 V, respectively, for phospholipid analysis. For MS/MS experiments, the collision gas pressure was typically 2.6–2.9 mTorr. To obtain fragmentation information on the Ag^+-phospholipid adducts, the dependence of collision energy on relative abundance was studied. Positive ions were detected scanning from 100 to 1000 amu with a total scan duration of 1 s. Profile data were recorded for 1 min (~60 scans) and averaged from analysis.

Samples were introduced either by direct liquid infusion (DLI) or HPLC. For DLI experiments, samples were introduced with a Harvard Apparatus (Cambridge, MA) syringe pump at a flow rate of 10 µL/min. For HPLC sample introduction, a Waters model 2690 Separation Model instrument was used. The HPLC was equipped with a Discovery octadecylsilane (ODS) column (4.6 × 250 mm, 5 µm; Supelco, Bellefonte, PA) and operated with a mobile phase of methanol/water (95:5, vol/vol) at a flow rate of 1 mL/min. A splitting tee after the column permitted 240 µL/min to be passed through an Applied Biosystems 785A programmable absorbance ultraviolet (UV) detector operating at 234 nm before entering the mass spectrometer. The remainder of the effluent was collected as waste.

Liquid chromatography (LC)–CIS–MS of phospholipid hydroperoxides. For these experiments, the hydroperoxides were isolated from the unoxidized phospholipid using analytical HPLC (methanol/water, 95:5, vol/vol, 1 mL/min). The oxidized fraction for PLPC (13.5–15.5 min) or SAPC (20–28 min) was collected, concentrated, and analyzed by LC–CIS–MS. A stock solution of the oxidized lipid was prepared (1–1.25 mg/mL) and 25–50 µL of the solution was injected per analysis. Offset voltages for selected reaction-monitoring (SRM) experiments, 28–33 eV, were determined by optimization in DLI experiments.

Studies on PLPC, autoxidation. To a solution of PLPC (25 mg, 0.033 mmol) in 2.5 mL of CH_2Cl_2 was added 10 mol% 2,2,5,7,8-pentamethyl-6-chromanol (PMC; 73 µL of a 10 mg/mL stock solution). The solution was evaporated to dryness under vacuum so that the mixture formed a thin layer on the inside of a 10-mL round-bottomed flask. The flask was then heated to 37°C and exposed to an atmosphere of dry air. After 24 h, the mixture was dissolved in benzene and BHT (~1–2 mg) was added to stop the reaction. TLC of the product mixture indicated the formation of hydroperoxides. The hydroperoxide products were then analyzed by HPLC.

HPLC analysis of the PLPC hydroperoxides (PLPC-OOH). In order to analyze the peroxidation products of PLPC, it was first necessary to optimize HPLC conditions for their separation. During the optimization process, two different C-18 analytical columns were tried; one was a Supelcosil column (4.6 × 250 mm, 5 μm; Supelco) while the other was a Discovery column (4.6 × 250 mm, 5 μm; Supelco). Mobile phases used in the optimization were mixtures of varying concentrations of methanol, hexanes, and water containing varying concentrations (0–25 mM) of ammonium acetate or choline chloride. The optimal conditions, which gave the best separation and peak shape, were obtained using the Discovery C-18 analytical column with a mobile phase of simply methanol/water (95:5, vol/vol) at a flow rate of 1 mL/min. Analysis of the PLPC-OOH using these HPLC conditions showed the formation of two major fractions at t_R = 14.04 and 14.97 min. Each fraction was collected, concentrated, and rechromatographed to ensure its purity. The fractions were then converted to the corresponding methyl hydroxyoctadecadienoates (HODE), which were previously characterized (23), for identification.

Conversion of PLPC-OOH to methyl HODE. Methyl hydroperoxyoctadecadienoates (HPODE) were prepared as described previously (23). This mixture of hydroperoxides and the isolated fractions of PLPC-OOH were treated with an excess of triphenylphosphine (PPh_3) to generate the corresponding alcohols (PLPC-OH). To convert the alcohols to the methyl esters, each isolated fraction was dissolved in benzene (0.5 mL) and treated with an excess of NaOMe (1 mL of a 0.5 M solution in methanol) for 2 h. The reactions were worked up by the addition of deionized water (5 mL) and acetic acid (100 μL). The aqueous layer was extracted with hexanes (2 × 5 mL). The combined organic layers were dried, concentrated, and analyzed by HPLC. To analyze the methyl HODE, the HPLC was equipped with two tandem Ultrasphere silica columns (4.6 × 250 mm, 5 μm; Beckman, Fullerton, CA). The compounds were eluted with 0.6% 2-propanol in hexanes containing 0.1% acetic acid at a flow rate of 3 mL/min. The elution order of the methyl HODE from the standard mixture corresponds to that previously reported (24). Injection of the transesterified fractions showed that phospholipid hydroperoxides **1**, **2**, and **3** elute from the HPLC in the first fraction, t_R = 14.04 min. Injection of the transesterified fractions showed that the second fraction, t_R = 14.97 min, contains phospholipid hydroperoxide **4**. ^{1}H NMR of each phospholipid fraction and LC–CIS–MS experiments support this assignment.

^{1}H NMR of the PLPC-OOH. ^{1}H NMR spectra were taken on each of the isolated PLPC-OOH fractions. The vinyl region of the spectrum taken on the first fraction consists of signals at δ 5.45 (*m*, 1H), 5.62 (*m*, 1H), 5.99 (*m*, 1H), and 6.51 (*m*, 1H). These signals are consistent with those signals previously reported for the *cis,trans* isomers of oxidized cholesteryl linoleate (25). The spectrum of this fraction also contains small signals that correspond to the signals due to the *trans,trans* isomer. These signals at δ 5.55 (*m*, 1H), 5.72 (*m*, 1H), 6.03 (*m*, 1H), and 6.21 (*m*, 1H) are the major vinyl signals observed in the second fraction and are consistent with those signals reported for the *trans,trans* isomers of oxidized cholesteryl linoleate. The data support the assignment of compounds **1**, **2**, and **3** in the first fraction, and the assignment of compound **4** to the second.

Studies on SAPC, autoxidation. To a solution of SAPC (25 mg, 0.029 mmol) in 2.5 mL of CH_2Cl_2 was added 10 mol% PMC (64 μL of a 10 mg/mL stock solution). The solution was evaporated to dryness under vacuum so that the mixture formed a thin layer on the inside of a 10-mL round-bottomed flask. The flask was then heated to 37°C and exposed to an atmosphere of dry air. After 24 h, the mixture was dissolved in benzene and BHT (~1–2 mg) was added to stop the reaction. Analytical HPLC (Discovery line ODS column, methanol/water, 95:5, vol/vol, 1 mL/min, λ = 234 nm) indicated the formation of six major fractions. These fractions were then identified by LC–CIS–MS: **I**, **11**, t_R = 20.93 min; **II**, **9**, t_R = 22.03 min; **III**, **10**, t_R = 22.78 min; **IV**, **7**, t_R = 24.21 min; $\mathbf{V_a}$, **(*R,R*)-8**, t_R = 24.89 min; $\mathbf{V_b}$, **(*S,R*)-8**, t_R = 25.28 min; and **VI**, **6**, t_R = 27.33 min.

Studies on fractions V_a and V_b. Fractions $\mathbf{V_a}$ and $\mathbf{V_b}$ were isolated from each other using analytical HPLC (Discovery ODS column, methanol/water, 93:7, vol/vol., 1 mL/min, λ = 234 nm). The fractions were concentrated and rechromatographed to ensure their purity. The compounds were then converted to the corresponding methyl HETE in an analogous procedure to that described above for the PLPC-OOH. The transesterified products were purified using the same analytical HPLC system described for the methyl HODE. The collected products were concentrated for analysis by chiral HPLC equipped with a Chiralpak AD column (4.6 × 250 mm, 10-mm; Chiral Technologies, Exton, PA) using a mobile phase of hexanes/methanol (100:2, vol/vol) at a flow rate of 1 mL/min (26). Coinjection of each transesterified fraction with a racemic mixture of methyl 9-HETE confirmed the elution order. The transesterified product from fraction $\mathbf{V_a}$, t_R = 8.65 min, was found to correspond to the *R* enantiomer of methyl 9-HETE; the transesterified product from fraction $\mathbf{V_b}$, t_R = 9.08 min, was found to correspond to the *S* enantiomer of methyl 9-HETE.

*Determination of the elution order of methyl (9*R*)- and (9*S*)-hydroxy-5(*Z*),8(*E*),11(*Z*),14(*Z*)-eicosatetraenoate in chiral HPLC analyses, autoxidation of eicosatetraenoic acid (arachidonic acid).* In a round-bottomed flask, arachidonic acid (500 mg, 1.64 mmol) was dissolved in 1,4-cyclohexadiene (3.2 mL, 34 mmol) and benzene (5 mL) to give a solution 0.20 M in lipid and 4.2 M in 1,4-cyclohexadiene. Di-*tert*-butylhyponitrite (~3–5 mg) was added to the reaction, and the flask contents were allowed to stir at 37°C for 24 h. At this time, TLC indicated the formation of hydroperoxide products. BHT (~2 mg) was added to stop the reaction. Benzene and 1,4-cyclohexadiene were removed by vacuum, and the mixture was rediluted in hexanes for separation by semipreparative HPLC [Dynamax-60 A silica 83-121-C column (Rainin Instrument Co. Inc., Woburn, MA), 21.4 × 250 mm, 8 μm, 2% 2-propanol in hexanes with 1% acetic acid, 10 mL/min]. The elution order for the HPETE is known (27). 9-HPETE was collected, t_R = 32 min, into a round-bottomed flask containing BHT (~2 mg). The solvent was removed *in vacuo,* and the racemic mixture of 9-HPETE was converted to the corresponding alcohols by treatment with excess PPh_3 in Et_2O.

Synthesis of methyl 9-hydroxy-5(Z),8(E),11(Z),14(Z)-eicosatetraenoate (methyl 9-HETE). The crude alcohol, 9-HETE, was dissolved in Et_2O (1 mL) and treated with an excess of diazomethane generated in a Micro Diazomethane Generator (Ace Glass, Inc.) with 1-methyl-3-nitro-1-nitrosoguandine as the precursor. The reaction was stirred at 0°C for 2 h. The reaction mixture was allowed to sit open to the air in a hood for 30 min to allow evaporation of any excess diazomethane. The ether was removed *in vacuo,* and the methyl ester was purified by analytical HPLC (two tandem Beckman silica columns, 1.6% 2-propanol in hexanes, 1 mL/min, $\lambda = 234$ nm).

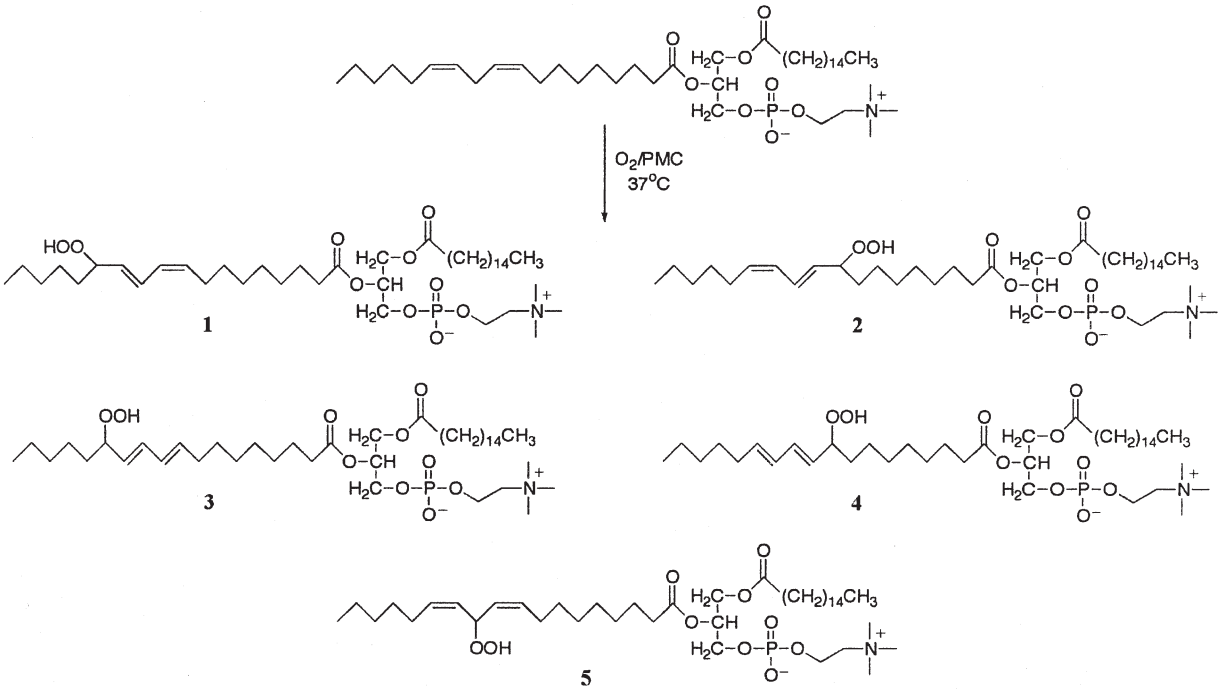
Separation of racemic methyl 9-HETE by chiral HPLC (26). The racemic methyl 9-HETE was separated using semi-preparative chiral HPLC. The HPLC was equipped with a Chiralpak AD column (10 × 250 mm, 10-mm; Chiral Technologies) using a mobile phase of hexanes/methanol (100:2, vol/vol) at a flow rate of 3 mL/min. Each enantiomer was collected, concentrated, and reinjected to ensure its purity: $\mathbf{V_a}$, t_R = 13.82 min; and $\mathbf{V_b}$, t_R = 14.47 min.

Circular dichroism (CD) spectroscopy (28). Each purified enantiomer was dissolved in 50 µL of dry acetonitrile. One microliter of 1,8-diazabicyclo[5.4.0]undec-1-ene and a few grains of 1-(2-naphthoyl)imidazole were added to the solution. The reaction was kept at room temperature overnight. The solvent was evaporated, and the residue was redissolved in CH_2Cl_2 (2 mL). The solution was washed twice with water (1 mL), and the CH_2Cl_2 was removed. The naphthoate derivatives were purified by analytical HPLC (two tandem Beckman silica columns, 1% 2-propanol in hexanes, 1 mL/min, $\lambda = 239$ nm). For CD spectroscopy, the purified naphthoate derivatives were dissolved in 400 µL of dry acetonitrile. CD was measured with a Jasco J-720 spectropolarimeter (cell volume 400 µL; cell path length 1 mm). The configuration of the collected enantiomer $\mathbf{V_a}$ was determined to be *R*, whereas the configuration of the collected enantiomer $\mathbf{V_b}$ was determined to be *S*.

RESULTS AND DISCUSSION

As previously reported (10), the peroxidation products, **1–4**, that form when a neat film of PLPC is incubated in an atmosphere of air at 37°C are from linoleoyl side-chain oxidation (Scheme 1). The mechanism by which the four peroxidic products of linoleate form is well understood (7–9). Initial hydrogen atom extraction at the C-11 position on the linoleoyl side chain yields a pentadienyl radical. A molecule of oxygen then adds to this stable radical at either the C-9 or the C-13 positions to give *cis,trans* peroxyl radicals that, in the presence of a good hydrogen atom donor, will be trapped as the *cis,trans* hydroperoxides **2** and **1**, respectively (in the presence of large amounts of a good hydrogen atom donor, such as α-tocopherol, the 11-hydroperoxide of linoleate is formed; 29). In the absence of hydrogen atom donors, the *trans,trans* hydroperoxides **3** and **4** form. The ratio of *cis,trans* to *trans,trans* products formed in a reaction mixture is a good measure of the competition between hydrogen atom abstraction from hydrogen donor molecules and β-fragmentation.

In our previous study, **1–4** were separated from unoxidized PLPC by HPLC, but all four phospholipid hydroperoxides eluted as one broad, tailing peak. There was no separation of the various hydroperoxide isomers (10). Our first goal in this study was to improve chromatography for the separation of phospholipid hydroperoxides by methods that were compatible with CIS–MS techniques. Therond *et al.* (24) achieved



SCHEME 1

good separation of the molecular species of soybean PC and their corresponding hydroperoxides, formed from incubation with soybean lipoxygenase, using an analytical Spherisorb C-18 column and a mobile phase of methanol/10 mM ammonium acetate (95:5, vol/vol) at 1 mL/min. Chromatography of a mixture of **1–4** on a Supelcosil C-18 column with a mobile phase of methanol/hexanes/water (100:5:5, by vol) containing 10 mM ammonium acetate gave sufficient separation of the PLPC-OOH from unoxidized PLPC, but there was little separation of the different isomers **1–4**. Better separation was achieved with a mobile phase of methanol/hexanes/water (100:1:5, by vol) containing 10 mM choline chloride. Using choline chloride as an ion-pairing agent resolved the tailing peak observed when ammonium acetate was used. The best separation of the phospholipid hydroperoxides was achieved using a newly marketed HPLC column, the Discovery C-18 column from Supelco, with a mobile phase of methanol/water (95:5, vol/vol) containing no ion-pairing agent.

With the optimal chromatographic conditions, two resolved hydroperoxide peaks with retention times (t_R) of 14.04 and 14.97 min were observed by UV detection at λ = 234 nm. To determine the elution order of **1–4**, compounds eluting in these two peaks were isolated. The collected phospholipid hydroperoxides in each peak were converted to the corresponding HODE methyl esters by reduction (PPh_3) and transesterification ($NaOCH_3$, CH_3OH). The HODE methyl esters are well characterized, and the chromatography of these compounds by normal-phase HPLC has been reported (23). This analysis showed that the first-eluting peak, t_R = 14.04 min, contained both of the 13-substituted hydroperoxides, **1** and **3**, and the 9-*cis,trans* hydroperoxide of PLPC, **2**. The second peak, t_R = 14.97 min, contained only the 9-*trans,trans* hydroperoxide, **4**.

It is possible to calculate the *cis,trans* to *trans,trans* product ratio based on the UV absorbance of each eluting peak, assuming that **3** is formed to the same extent as **4**, and that **1** is formed to the same extent as **2.** (This assumption is made based on the calculated extinction coefficients for the *trans,cis* and *trans,trans* hydroperoxides of methyl linoleate; 30.) By using a mixture of hydroperoxides **1–4** that were generated from a standard oxidation of PLPC without a hydrogen atom donor present, the *cis,trans* to *trans,trans* ratio was calculated to be 0.43 by HPLC under the optimal conditions described above. A portion of this oxidation mixture was converted to the corresponding HODE methyl esters, and the *cis,trans* to *trans,trans* ratio was determined by standard normal-phase HPLC–UV analysis. This number was also found to be 0.43, comparable with that calculated using the intact phospholipid hydroperoxides. Being able to determine the product ratio from the intact phospholipid hydroperoxides eliminates the need for converting **1–4** to the methyl esters and may be beneficial in future oxidation studies of complex phospholipid mixtures.

Having developed a chromatographic method that resolved some of the PLPC-OOH isomers, we explored analytical methods that would permit identification of oxidized phospholipid molecular species without conversion or derivatization. CIS–MS has proved to be useful for obtaining structural information for several classes of cholesteryl ester peroxides and hydroperoxides (22), and we therefore turned to CIS–MS for the analysis of intact phospholipid hydroperoxides. In the first experiments, **1–4** were isolated from unoxidized PLPC by HPLC. The collected hydroperoxides were introduced into the mass spectrometer *via* DLI as a mixture in methanol with 4 equivalents of $AgBF_4$. The resulting CIS–MS spectrum is shown in Figure 1A. The dominant ions observed are the $[M + Ag]^+$ adducts at *m/z* = 896 and 898, formed from ^{107}Ag and ^{109}Ag isotopes that are present in a ratio of ~1:1.

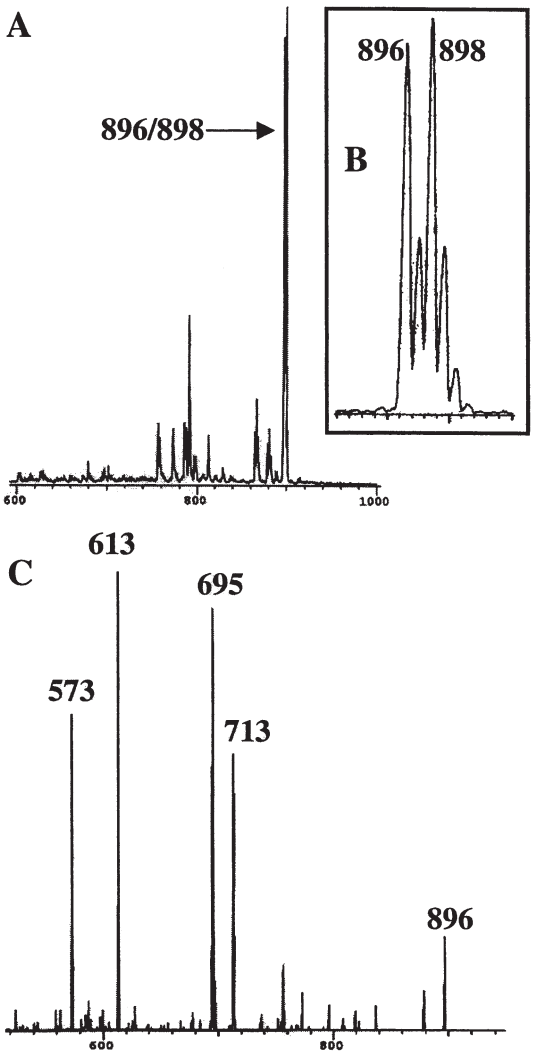
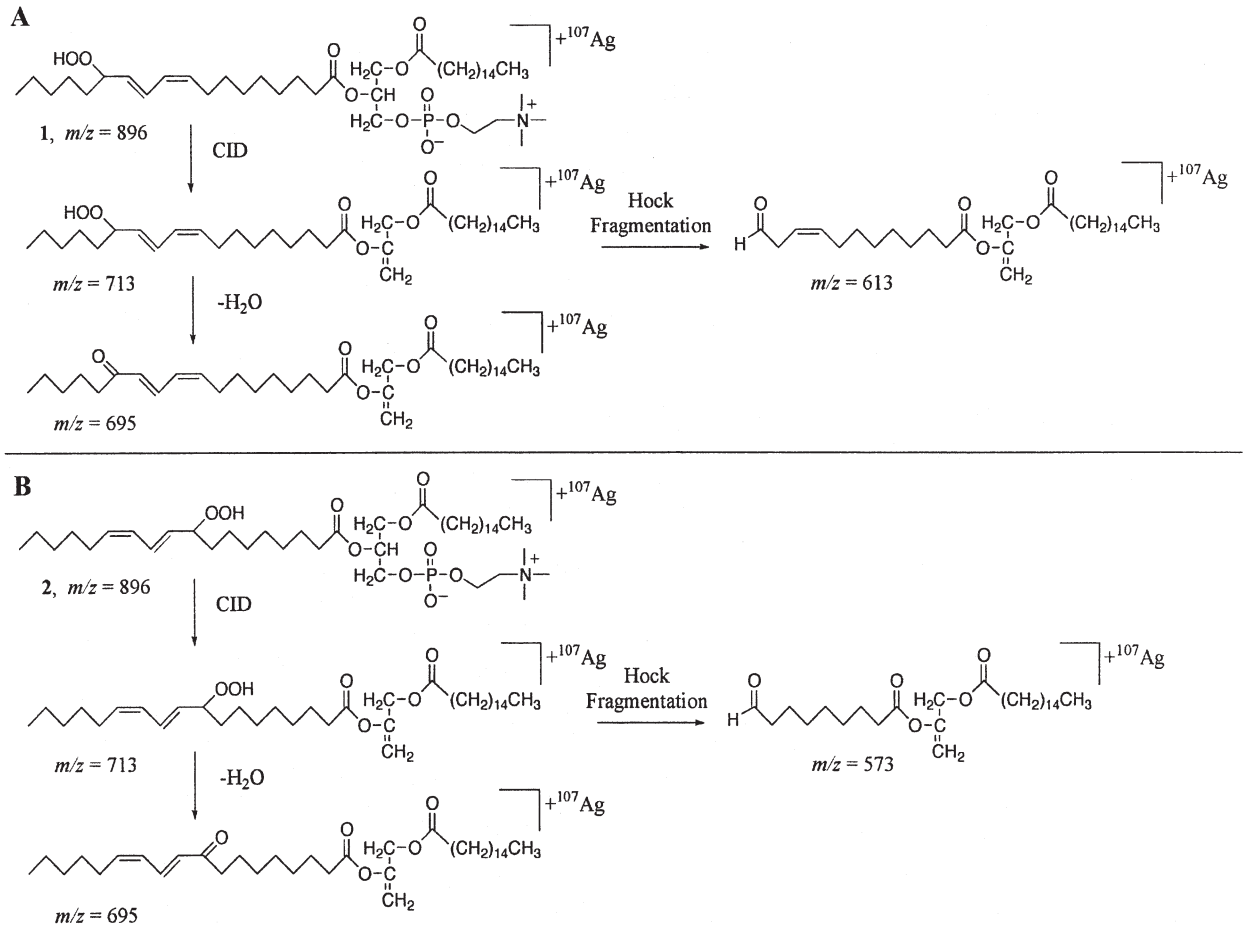


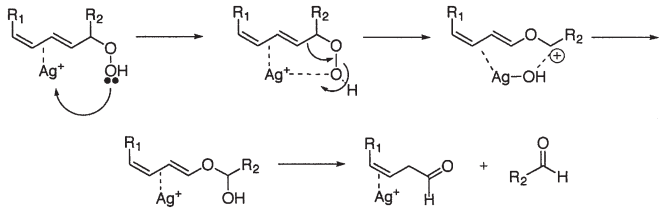
FIG. 1. Silver ion coordination ion-spray mass spectrum of a mixture of **1–4** obtained in direct liquid infusion experiments: (A) full scan, (B) blow-up of the $[M + Ag]^+$ region, and (C) collision-induced dissociation spectrum of *m/z* = 896.

CID experiments on the complex of **1–4** with $^{107}Ag^+$ at *m/z* = 896 gave fragment ions at *m/z* = 713, 695, 613, and 573 (Fig. 1C). The proposed structures for each of the fragment ions are shown in Scheme 2. Each fragment showed loss of the phosphocholine head group as a neutral loss of 183. The fragment at *m/z* = 713 $[M + Ag - 183]^+$ exhibits only loss of the head group, whereas the fragment at *m/z* = 695 $[M + Ag - 183 - H_2O]^+$, in



SCHEME 2

addition to head group loss, shows dehydration of the hydroperoxide on the side chain. We suggest that the fragments observed at *m/z* = 613 and 573 are from Hock fragmentation of the silver ion hydroperoxide complex. This fragmentation was the primary mechanism of cholesteryl ester hydroperoxide fragmentation in CIS–MS studies (22). This commonly observed fragmentation (or rearrangement) of lipid hydroperoxides is promoted by protic or Lewis acids in solution at moderate temperatures (31–34). In the collision cell of the mass spectrometer, this fragmentation is most likely catalyzed by the silver ion, a Lewis acid (Scheme 3). The Hock fragments provide valuable information about the position of the hydroperoxide on the linoleoyl side chain. The fragment ion observed at *m/z* = 613 results from collisional activation of the 13-substituted hydroperoxides **1** and **3**, while the fragment at *m/z* = 573 results from activation of the 9-substituted hydroperoxides **2** and **4**.



SCHEME 3

Coupling HPLC and LC–CIS–MS was achieved by either postcolumn mixing of 0.50 mM $AgBF_4$ in methanol with the HPLC effluent or by addition of $AgBF_4$ to the HPLC mobile phase to yield a 0.15 mM solution. Chromatography was carried out using the new Supelco Discovery C-18 analytical column with a mobile phase of methanol/water (95:5, vol/vol) at a flow rate of 1 mL/min. A flow splitter was inserted online so that 240 μL/min was directed through a UV detector and into the mass spectrometer while 760 μL/min was collected as waste. Typical chromatograms from the mass spectrometer for a mixture of PLPC-OOH are shown in Figure 2. Panel A shows the total ion current chromatogram for the silver ion adducts. Panel B shows the chromatogram of the 13-substituted hydroperoxides **1** and **3** that co-elute. Panel C shows the chromatogram of the 9-substituted hydroperoxides **2** and **4** that separate under these chromatographic conditions.

These chromatograms result from the mass spectrometer being operated in the SRM mode. In this mode, a specific precursor-to-product mass conversion produced at a characteristic energy in the collision cell is monitored. SRM separates the differently substituted hydroperoxides based on the difference in their Hock fragments. The elution pattern of the hydroperoxides in these SRM experiments is consistent with the elution order determined by separation of the hydroper-

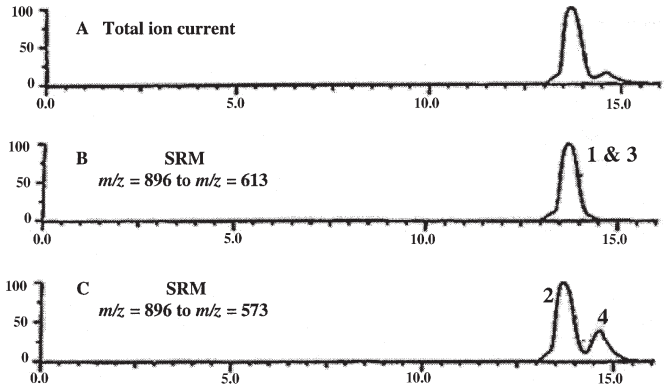


FIG. 2. Chromatograms of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine oxidation mixture formed when oxidized in the presence of 0.1 equivalents of pentamethylchromanol: (A) high-pressure liquid chromatography–coordination ion-spray–mass spectrometry (HPLC–CIS–MS) total ion current is a sum of (B) and (C); (B) HPLC–CIS–MS in selected reaction monitoring (SRM) mode for $m/z = 896 \rightarrow 613$; (C) HPLC–CIS–MS in selected reaction monitoring mode for $m/z = 896 \rightarrow 573$.

oxides by HPLC and subsequent conversion to the corresponding HODE methyl esters.

LC–CIS–MS experiments were also used to identify the 11-substituted hydroperoxide of PLPC, **5** (Fig. 3). The 11-substituted hydroperoxides of methyl linoleate (29) and cholesteryl linoleate (35) were only observed when the oxidation was performed in the presence of large amounts of α-tocopherol. Thus, to identify **5**, PLPC was oxidized as a thin film with 0.75 equivalents of α-tocopherol at 37°C for 24 h. By operating the mass spectrometer in SRM mode and monitoring for the conversion of **5** to its two possible Hock fragments at $m/z = 599$ and 587, the 11-substituted hydroperoxide was identified as the peak eluting at 13.77 min in Figure 3.

With the chromatographic and mass spectrometric techniques developed for analyzing the phospholipid hydroperoxides from PLPC, the hydroperoxides from another phospholipid, SAPC, were studied. The oxidation mixture from SAPC is more complex than that from PLPC because there are more sites of unsaturation on the arachidonoyl side chain. The six hydroperoxides, **6–11**, obtained from oxidizing a neat film of SAPC in the presence of a good hydrogen atom donor, PMC, are shown in Scheme 4. These products, which we previously reported (10), are formed in an analogous way to the hydroperoxides derived from PLPC.

A typical UV chromatogram obtained for **6–11** at λ = 234 nm is shown in Figure 4A, and the corresponding alcohols gave the chromatogram shown in Figure 5. The phospholipid hydroperoxides were then analyzed using LC–CIS–MS with the mass spectrometer being operated in both full-scan and SRM modes. It is of importance to note here that the starting phospholipid used in these studies was purchased from the Sigma Chemical Company. In our initial experiments, SAPC

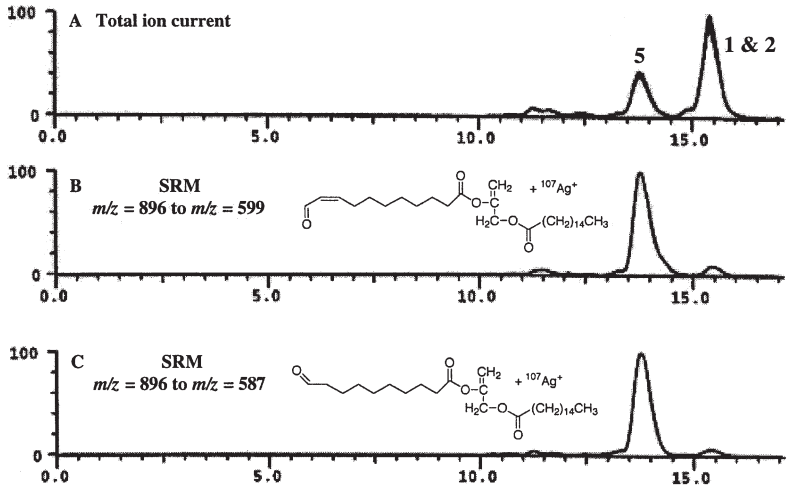
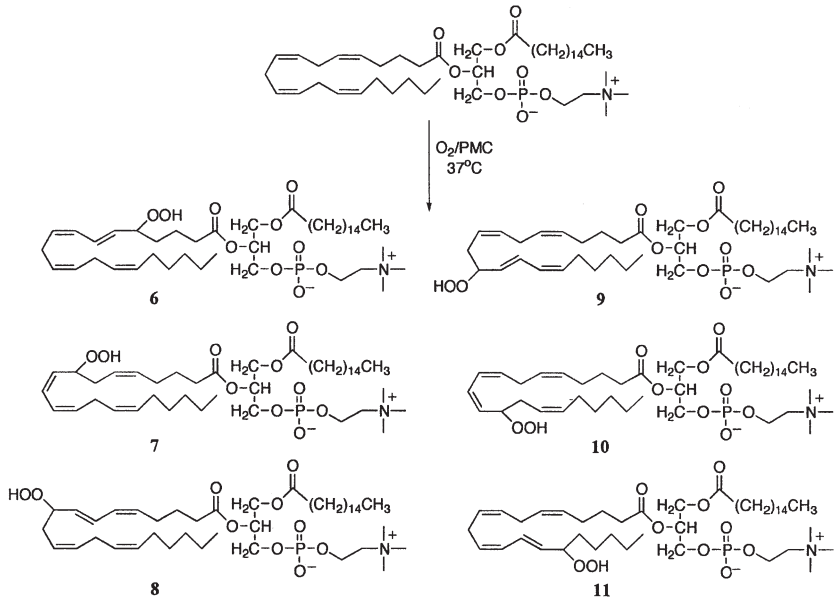


FIG. 3. Chromatograms of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine oxidation mixture formed when oxidized in the presence of 0.75 equivalents of α-tocopherol: (A) HPLC–CIS–MS total ion current is a sum of (B), (C), and the SRM experiments monitoring for the 9- and 13-hydroperoxides (as in Fig. 2); (B) HPLC–CIS–MS in SRM mode for $m/z = 896 \rightarrow 599$; (C) HPLC–CIS–MS in SRM mode for $m/z = 896 \rightarrow 587$. For abbreviations see Figure 2.



SCHEME 4

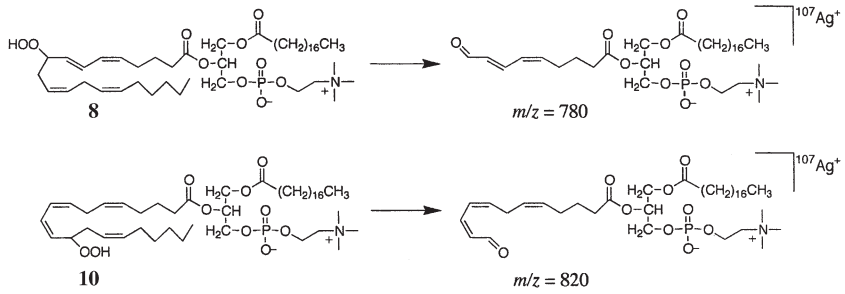
purchased from Avanti Polar Lipids was used, and a much more complex chromatogram was obtained. We speculate that this complexity was due to oxidation of 1-arachidonyl-2-stearoyl-*sn*-glycero-3-phosphatidylcholine, a positional isomer of SAPC present in the Avanti phospholipid. Avanti reports that the positional purity of their phospholipids can be as low as 80%, but Sigma reports a positional purity of 97%.

In scanning from *m/z* = 600 through 1000, the dominant ions in the LC–CIS–MS spectra for the phospholipid hydroperoxides **6–11** were *m/z* = 948 and 950. The elution order of the phospholipid hydroperoxides from the HPLC was determined using LC–CIS–MS with the mass spectrometer being operated in SRM mode (Fig. 4B–G).

Hydroperoxides **6** and **11** were easily identified as peaks **VI** and **I**, respectively, in Figure 4A because their Hock fragments are unique. However, hydroperoxides **7** and **8**, identified by SRM as peaks **IV** and **V**, have identical Hock fragments, as do hydroperoxides **9** and **10**, identified as peaks **II** and **III**. These hydroperoxides were distinguished from each other by monitoring unique, less abundant fragments from **8** and **10** in SRM mode. These fragments at *m/z* = 780 and 820, shown in Scheme 5, result from cleavage of the C9-C10 bond α to the hydroperoxide in **8** and the C12-C13 bond α to the hydroperoxide in **10**, respectively. This fragmentation pattern, analogous to that observed in the analysis of HETE and HETE PC by negative-ion FAB–MS and ESI–MS (13,16,36,37), helped identify **8** as peak **V** and **10** as peak **III**.

In the UV chromatogram (Fig. 4A), peak **V** actually appears to be two closely eluting peaks. This same pattern for peak **V** is observed in the SRM experiments monitoring for **8** [9-hydroperoxyeicosatetraenoyl-*sn*-glycero-3-phosphatidylcholine (9-HPETE PC)]. This suggested that both of these peaks, $\mathbf{V_a}$ and $\mathbf{V_b}$, are 9-HPETE PC and not a co-eluting contaminant. Unoxidized phospholipids are chiral molecules with a stereogenic center at C_2 of the glycerol backbone; the configuration of this center is *R*. When the phospholipid is oxidized to the hydroperoxide, diastereomers are formed because oxygen addition can occur to either face of the pentadienyl radical, and we suspected that these diastereomers of 9-HPETE PC were being separated as peaks $\mathbf{V_a}$ and $\mathbf{V_b}$.

To test the hypothesis that we were observing separation of diastereomers, compounds eluting in peaks $\mathbf{V_a}$ and $\mathbf{V_b}$ were isolated by HPLC and converted to the corresponding methyl HETE. Methyl HETE contain only one stereogenic center at the position of oxygen addition and exist as enantiomers that can be separated using chiral HPLC as reported



SCHEME 5

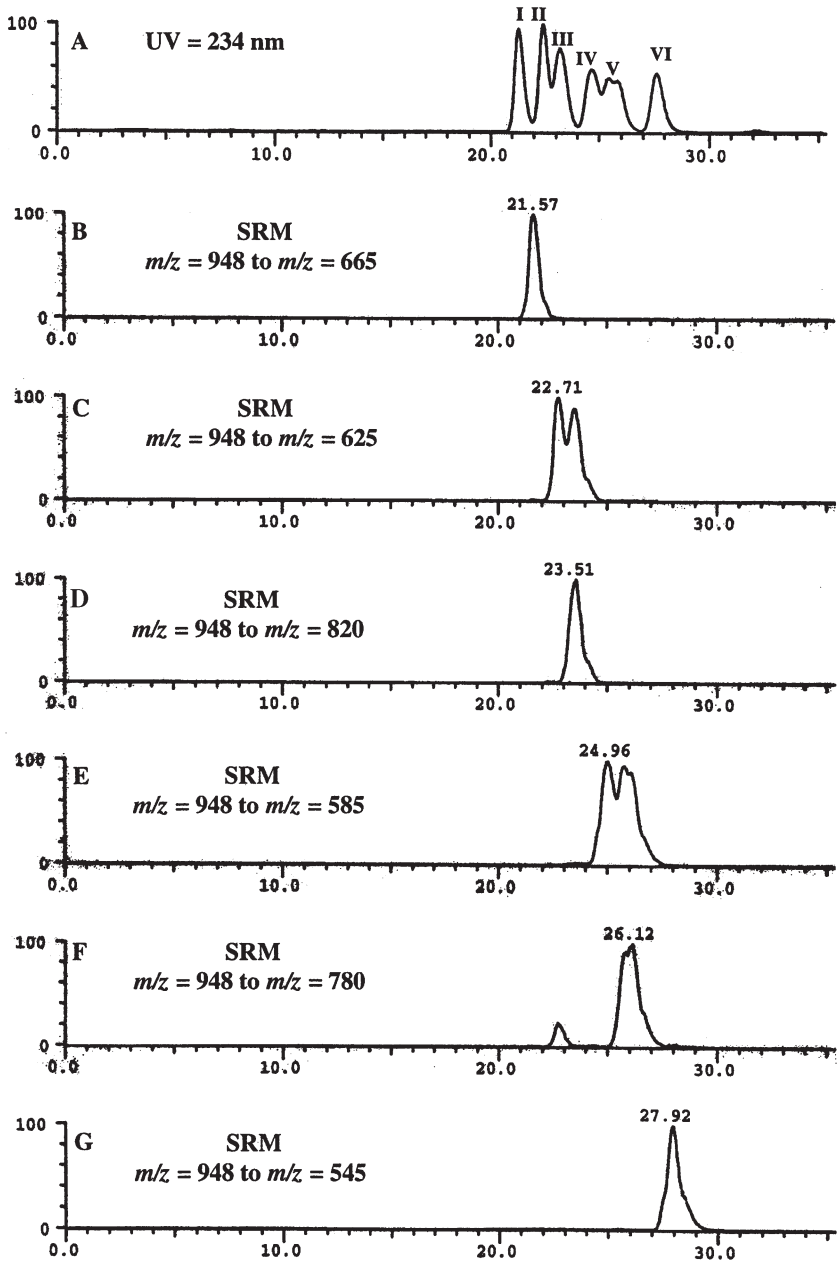


FIG. 4. Chromatograms of the product mixture obtained from a 24-h oxidation of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine containing 0.1 equivalents of pentamethylchromanol (mobile phase, methanol/water, 95:5, vol/vol; Discovery C-18 analytical column, Supelco, Bellefonte, PA): (A) ultraviolet detection at λ = 234 nm: **I** = 15-HPETE PC, **II** = 11-HPETE PC, **III** = 12-HPETE PC, **IV** = 8-HPETE PC, **V** = 9-HPETE PC, **VI** = 5-HPETE PC; (B–G) HPLC-CIS-MS in SRM mode. HPETE, hydroperoxyeicosatetraenoic acid; PC, phosphatidylcholine; for other abbreviations see Figure 2.

by Schneider *et al.* The methyl esters derived from collected peaks $\mathbf{V_a}$ and $\mathbf{V_b}$ were analyzed using an analytical Chiralpak AD column with a mobile phase of hexanes/methanol (100:2, vol/vol). Comparison with a standard mixture of racemic methyl 9-HETE supported the notion that the methyl ester derived from peak $\mathbf{V_a}$ was one enantiomer of methyl 9-HETE, and the methyl ester derived from peak $\mathbf{V_b}$ was the other enantiomer (Fig. 6). While it is of importance to report that the diastereomers of 9-HPETE PC are separable by RP-HPLC, we have no explanation for this fact of nature observed for the 9-HPETE PC and not the other HPETE PC.

To determine the elution order of the methyl 9-HETE enantiomers so that configurations could be assigned to the diastereomers of **8** eluting as peaks $\mathbf{V_a}$ and $\mathbf{V_b}$, it was necessary to synthesize derivatives of the compounds and determine their absolute configuration using circular dichroism (CD) spectroscopy. CD spectroscopy uses the interaction of two chromophores at the chiral center in order to define absolute config-

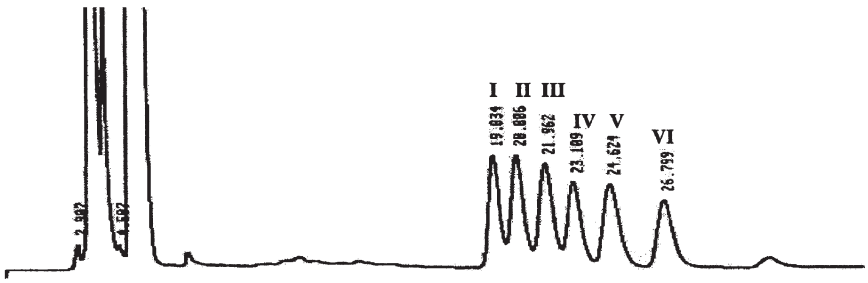


FIG 5. HPLC chromatogram of 1-stearoyl-2-arachidonyl-*sn*-glycero-3-phosphatidylcholine alcohol (SAPC-OH) formed by reduction of the SAPC-OOH (Scheme 4, Fig. 4) with triphenylphosphine. The elution order of the alcohols is identical to that of the hydroperoxides. For abbreviation see Figure 2.

uration. Conversion of the isolated 9-HETE methyl esters to their 2-naphthyl esters gave derivatives that provide information about the absolute configurations of the compounds (28).

The derivative of the first-eluting 9-HETE methyl ester enantiomer showed a negative first Cotton effect (CE) at λ = 243 nm ($\Delta\varepsilon$ −50.8) and a positive second CE at λ = 226 nm ($\Delta\varepsilon$ +41.6) (By definition, if the chirality of the electronic transition moments of the chromophore of highest wavelength to the chromophore of the lowest wavelength is counterclockwise when the molecule is represented in the Newman projection, the CD shows a negative first and a positive second CE and vice versa). Thus, this enantiomer was assigned an absolute configuration of *R*. The derivative of the later-eluting enantiomer showed a positive first CE at λ = 243 nm ($\Delta\varepsilon$ +19.6) and a negative second CE at λ = 226 nm ($\Delta\varepsilon$ −16.1) and was assigned an absolute configuration of *S*. Using this information, the first-eluting diasteromer of **8** ($\mathbf{V_a}$) has the *R,R* configuration, and the second-eluting diastereomer ($\mathbf{V_b}$) is *S,R*.

CIS–MS is a powerful technique for the identification of phospholipid hydroperoxides. Also, by using the new Supelco Discovery C-18 columns and a mobile phase of only methanol and water, separation of phospholipid hydroperoxide isomers has been achieved for the first time. Currently, we are using these analytical techniques to identify phospholipid hydroperoxides formed in oxidations of low-density lipoproteins.

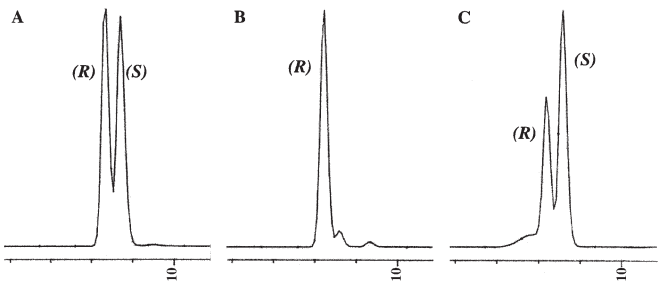


FIG 6. Chiral HPLC chromatograms of the 9-HETE methyl ester enantiomers (mobile phase, hexanes/methanol, 100:2, vol/vol; Chiralpak AD analytical column, Chiral Technologies, Exton, PA; UV detection at λ = 234 nm): (A) racemic methyl 9-HETE; (B) methyl 9-HETE derived from fraction $\mathbf{V_a}$; (C) methyl 9-HETE derived from fraction $\mathbf{V_b}$. HETE, hydroxyeicosatetraenoic acid; for other abbreviation see Figure 2.

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