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Liquid chromatography/mass spectrometry analysis of bifunctional electrophiles and DNA adducts from vitamin C mediated decomposition of 15-hydroperoxyeicosatetraenoic acid

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Reactive oxygen species convert the ω-6 polyunsaturated fatty acid arachidonic acid into 15-hydroperoxy-5,8,11,13-(*Z,Z,ZE*)-eicosatetraenoic acid (15-HPETE). Cyclooxygenases and lipoxygenases can also convert arachidonic acid into 15-HPETE. Vitamin C mediated decomposition of 15(*S*)-HPETE to protein- and DNA-reactive bifunctional electrophiles was examined by normal-phase liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC/APCI-MS). The individual bifunctional electrophiles, *trans*-4,5-epoxy-2(*E*)-decenal (*t*-EDE), *cis*-4,5-epoxy-2(*E*)-decenal (*c*-EDE), 4-oxo-2(*E*)-nonenal (ONE), and 4-hydroxy-2(*E*)-nonenal (HNE), exhibited protonated molecules at *m/z* 169, 169, 155, and 157, respectively. The MH^+ ion at *m/z* 173 for 4-hydroperoxy-2(*E*)-nonenal (HPNE) was very weak with an ion corresponding to the loss of OH at *m/z* 156 as the major ion in the APCI mass spectrum. The bifunctional electrophiles were all separated under normal-phase LC conditions. All five bifunctional electrophiles were formed when 15-HPETE was treated with vitamin C. The LC/MS-based methodology showed that *t*-EDE was the major bifunctional electrophile formed during vitamin C mediated 15(*S*)-HPETE decomposition. Stable isotope dilution LC/MS studies revealed that this did not result in the formation of increased levels of unsubstituted etheno-dGuo adducts in calf thymus DNA when compared with 13(*S*)-hydroperoxy-9,10-(*Z,E*)-octadecadienoic acid [13(*S*)-HPODE], a lipid hydroperoxide derived from linoleic acid. However, the formation of heptanone-etheno-dGuo adducts in calf thymus DNA was reduced when compared with the 13(*S*)-HPODE. This was attributed to the reduced formation of ONE from 15-HPETE when compared with its formation from 13-HPODE. In contrast to reactions with dGuo or DNA conducted using 13(*S*)-HPODE, no carboxy-containing adducts were observed with 15(*S*)-HPETE.

Lipid peroxidation has been implicated in the degenerative diseases of aging, specifically cancer, cardiovascular disease, and neurodegeneration. Lipid peroxidation has been described as the ''oxidative deterioration of membrane lipids''.[1] It can be initiated enzymatically or by reactive oxygen (ROS)-mediated action on polyunsaturated fatty acids (PUFAs).[2] Enzymes involved in lipid peroxidation include the lipoxygenases (LOXs)[3] and the cyclooxygenases (COXs).[4] Both LOX and COX enzymes abstract a pro-(*S*) hydrogen from a *bis*-allylic methylene group in the PUFA, followed by a double-bond rearrangement and antaraficial oxygen insertion.[5,6] 15-LOX reacts with linoleic acid, the major PUFA in cooking oils (such as safflower oil) and plasma, to produce 13(*S*)-hydroperoxy-9,10-(*Z,E*)-octadecadienoic acid [13(*S*)-HPODE; a prototypic ω-6 PUFA lipid hydroperoxide] while both COX-1 and COX-2 react with linoleic acid (although COX-2 reacts to a higher degree than COX-1) to produce primarily 9(*R*)-HPODE and 13(*S*)-HPODE.[7] ROS-mediated oxidation of linoleic acid results in the formation of racemic 13-HPODE as well as a number of other isomers.[8] In a number of previous studies, the decomposition of 13(*S*)-HPODE was examined. The major decomposition products were 4-hydroperoxy-2(*E*)-nonenal (HPNE), 4-oxo-2(*E*)-nonenal (ONE), 4-hydroxy-2(*E*)-nonenal (HNE), *trans*-4,5-epoxy-2(*E*)-decenal (*t*-EDE), *cis*-4,5-epoxy-2(*E*)-decenal (*c*-EDE), and 9,12-dioxo-10(*E*)-dodecenoic acid (DODE) (Scheme 1). These bifunctional electrophiles are formed by three distinct pathways (Scheme 1). The first pathway involves α-cleavage of an alkoxy radical and results in formation of EDE.[9,10] The second pathway involves formation of HPNE, which undergoes reduction into HNE[11,12] and ONE.[11,13,14] The third

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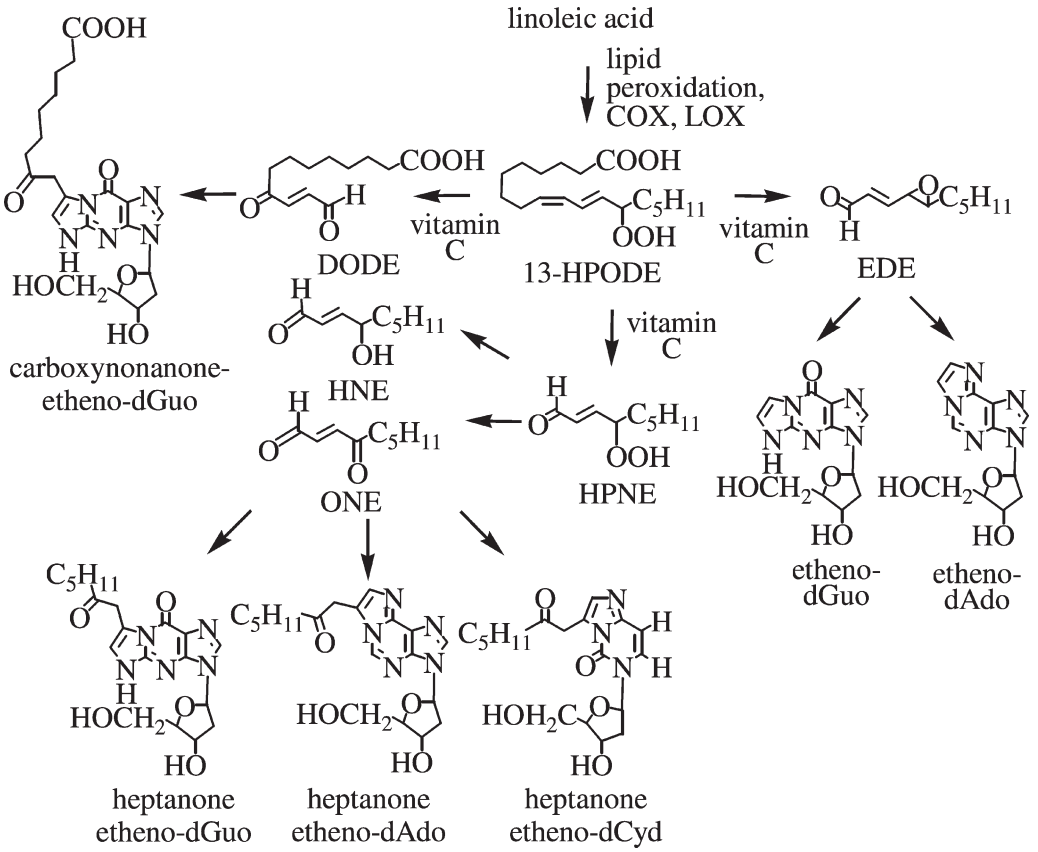

Scheme 1. Vitamin C mediated decomposition of 13-HPODE to bifunctional electrophiles and etheno-DNA adducts.

pathway most likely involves the intermediate formation of 9-hydroperoxy-12-oxo-10(*E*)-dodecenoic acid, which undergoes reduction into DODE[15] analogous to HPNE.[11,12]

In a series of studies, ONE was shown to be the precursor in the formation of heptanone-1,N^6-etheno-2′-deoxyadenosine (dAdo),[16] heptanone-1,N^2-etheno-2′-deoxyguanosine (dGuo),[17,18] and heptanone-3,N^4-etheno-2′-deoxycytidine[19] adducts and that EDE was the precursor in the formation of unsubstituted 1,N^2-etheno-dAdo and 1,N^6-etheno-dGuo adducts.[10] DODE was recently shown to form a carboxynonanone-etheno-dGuo adduct.[15]

Under normal physiological conditions, arachidonic acid is the major substrate for COX-1 and COX-2. For example it has a $k_{cat}/K_m = 19$ for COX-2 compared with a $k_{cat}/K_m = 2.8$ of linoleic acid for COX-2.[4] Arachidonic acid is the precursor to the formation of 15-hydroperoxy-5,8,11,13-(*Z*,*Z*,*ZE*)-eicosatetraenoic acid (15-HPETE) in addition to prostaglandins (PGs) and thromboxanes (TXs).[11] The resulting 15-HPETE is subsequently reduced to 15-HETE either by the peroxidase component of the COXs or by reducing factors in the cellular milieu.[4,5] 15-HETE generated from COX-1 consists of an almost racemic mixture of *R* and *S*-enantiomers,[20,21] whereas COX-2 produces primarily the 15*S*-enantiomer.[21] Arachidonic acid is also an excellent substrate for 15-LOX-1[22] and 15-LOX-2[23] where it produces exclusively 15(*S*)-HPETE. ROS-mediated arachidonic acid metabolism results in the formation of racemic 15-HPETE together with a number of other isomers.[7] We have recently developed chiral liquid chromatography/mass spectrometry (LC/MS) methodology to monitor the chirality of 15-HETE formation during cellular COX-mediated arachidonic acid metabolism.[24] Therefore, it is now possible to determine whether the 15-HETE is generated enzymatically and to distinguish between COX-1 and COX-2 pathways.

During cellular oxidative stress, reducing cofactors become depleted. Under these conditions, COX-mediated formation of lipid hydroperoxides such as 15-HPETE from arachidonic acid may become more important than the generation of 13-HPODE through ROS-mediated pathways. Furthermore, the up-regulation of COX-2 in various diseases, most specifically colon cancer,[25] can potentially further increase the formation of 15-HPETE. This could result in the formation DNA-reactive α,β-unsaturated bifunctional electrophiles in increased amounts and provide a genotoxic pathway, which is independent of PG and TX biosynthesis. We report the use of LC/MS to characterize the 15-HPETE-derived profile of aldehydic bifunctional electrophiles and the use of stable isotope dilution LC/tandem mass spectrometry (MS/MS) for quantitation of the etheno-dGuo DNA adducts that result.

EXPERIMENTAL

Materials

Acetic acid, ammonium acetate, L-ascorbic acid (vitamin C), activated calf thymus DNA, 2′-deoxyadenosine (dAdo), 2′-deoxyguanosine (dGuo), dichloromethane, DNAse I, EDTA, hydrochloric acid, lipoxidase (soybean), *N*,*N*-diisopropylethylamine,

2,3,4,5,6-pentafluorobenzyl bromide, sodium chloride, sodium sulfate, and zinc chloride were purchased from Sigma-Aldrich. (St. Louis, MO, USA). Arachidonic acid, 15(*S*)-HPETE, 4-hydroxy-2(*E*)-nonenal, and 13-HPODE were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Nuclease P1 and alkaline phosphatase (shrimp) were obtained from Roche Diagnostics (Indianapolis, IN, USA). 3-Morpholinopropanesulfonic acid (MOPS) was obtained from Fluka BioChemika (Milwaukee, WI, USA). Chelex-100 chelating ion-exchange resin (100–200 mesh size) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). HPLC-grade water, acetonitrile, diethyl ether, ethanol, hexane, methanol, 2-propanol, and tetrahydrofuran were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA). Gases were supplied by BOC Gases (Lebanon, NJ, USA). Transition metal ion free buffers and ONE were prepared as described previously.[13] Authentic *t*-EDE and *c*-EDE were prepared by homologation of *trans*-2-octenal.[10] The HPNE was prepared enzymatically from 3(*Z*)-nonenal.[26]

Mass spectrometry

Mass spectrometry for qualitative analysis of bifunctional electrophiles and DNA adducts was conducted with a Finnigan LCQ ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source in positive ion mode. Operating conditions 1 were as follows: vaporizer temperature, 450°C; heated capillary temperature, 150°C; with a discharge current of 5 μA applied to the corona needle. Nitrogen was used as the sheath (80 psi) and auxiliary (10 arbitrary units) gas to assist with nebulization. Full scanning analyses were performed in the range of *m/z* 100–800. Collision-induced dissociation (CID) experiments coupled with multiple tandem mass spectrometry (MS^n) employed helium as the collision gas. The relative collision energy was set at 20% of the maximum (1 V).

Mass spectrometry for quantitative analysis of bifunctional electrophiles and DNA adducts was conducted with a Finnigan TSQ Quantum Ultra AM spectrometer (Thermo Electron Corporation) equipped with an APCI source in the positive ion mode. Operating conditions 2 were as follows: vaporizer temperature, 450°C; heated capillary temperature, 160°C; with a discharge current of 15 μA applied to the corona needle. Nitrogen was used as the sheath (40 psi) and auxiliary (10 units) gas to assist with nebulization. CID experiments coupled with MS^n employed argon as the collision gas. Operating conditions 3 were as follows: vaporizer temperature, 550°C; heated capillary temperature, 180°C; with a discharge current of 18 μA applied to the corona needle. Nitrogen was used as the sheath (30 psi) and auxiliary (3 units) gas to assist with nebulization. CID experiments coupled with MS^n employed argon as the collision gas.

Liquid chromatography

Chromatography for LC/MS experiments for gradient systems 1 to 5 was performed using a Waters Alliance 2690 HPLC system (Waters Corp., Milford, MA, USA). An YMC C_{18} ODS-AQ column (250 × 4.6 mm i.d., 5 μm; Waters) was used in systems 1, 3 and 5 with a flow rate of 1.0 mL/min. Gradient system 2 included a Hi-Chrom silica column (250 × 4.6 mm i.d., 5 μm; Regis, Morton Grove, IL, USA) at a flow rate of 1 mL/min. Gradient system 4 employed two Hi-Chrom silica columns in series (250 mm × 4.6 mm i.d., 5 μm; Regis). For system 1, solvent A was 5 mM ammonium acetate in water, and solvent B was 5 mM ammonium acetate in acetonitrile. The linear gradient was as follows: 6% B at 0 min, 6% B at 3 min, 20% B at 9 min, 20% B at 13 min, 60% B at 21 min, 80% B at 22 min, and 80% B at 24 min. For system 2, solvent A was hexane/2-propanol (197:3, v/v) and solvent B was hexane/2-propanol (7:3, v/v). The gradient conditions were as follows: 3% B at 0 min, 3% B at 15 min, 85% B at 28 min, and 3% B at 30 min. For system 3, solvent A was tetrahydrofuran/methanol/water/acetic acid (25:30:44.9:0.1, v/v) and solvent B was methanol and water (9:1, v/v). Both solvents A and B contained 5 mM ammonium acetate. The gradient conditions were as follows: 70% B at 0 min, 70% B at 3 min, 100% B at 10 min, 100% B at 20 min, and 70% B at 23 min, followed by a 7-min equilibration time. For system 4, solvent A was hexane/isopropanol (197:3, v/v) and solvent B was hexane/2-propanol (70:30, v/v). The gradient conditions were as follows: isocratic elution with 19% B was conducted for 13 min at a flow rate of 0.8 mL/min. A linear gradient was then run to 25% B at 18 min at a flow rate of 1.0 mL/min. The separation was performed at ambient temperature. For system 5, solvent A was 5 mM ammonium acetate in water, and solvent B was 5 mM ammonium acetate in acetonitrile. The linear gradient was as follows: 6% B at 0 min, 6% B at 3 min, 20% B at 9 min, 20% B at 13 min, 60% B at 21 min, 80% B at 22 min, and 80% B at 24 min. The separation was performed at ambient temperature.

LC/UV analysis was conducted using gradient system 6 on a Hitachi L-6200A Intelligent pump equipped with a Hitachi L4000 UV detector (Hitachi, San Jose, CA, USA). The separation employed a Phenomenex Synergi 4u polar RP column (250 × 4.6 mm i.d., 4 μm). Solvent A was 5 mM ammonium acetate in water and solvent B was 5 mM ammonium acetate in acetonitrile. The gradient was as follows: 6% B at 0 min, 6% B at 3 min, 20% B at 9 min, 20% B at 10 min, 80% B at 12 min, 80% B at 15 min, 6% B at 17 min, and 6% B at 25 min. The flow rate was 1 mL/min. The separation was performed at ambient temperature. Gradient system 7 was performed on a Hitachi L2100 pump equipped with a L2400 UV detector (Hitachi). The separation employed an XTerra C18 column (250 × 4.6 mm i.d., 5 μm). Solvent A was 5 mM ammonium acetate in water and solvent B was 5 mM ammonium acetate in acetonitrile. The gradient was as follows: 6% B at 0 min, 6% B at 3 min, 20% B at 9 min, 20% B at 13 min, 40% B at 22 min, 70% B at 27 min, 80% B at 28 min, 80% B at 32 min, and 6% B at 34 min. The flow rate was 1.0 mL/min. The separation was performed at ambient temperature.

MS analysis of authentic bifunctional electrophiles

MS and MS/MS analyses were performed by direct infusion atmospheric pressure chemical ionization ion trap mass spectrometry (APCI-MS). MS and MS/MS spectra were accumulated for periods of 2–3 min. The solutions of authentic bifunctional electrophiles (*t*-EDE and *c*-EDE, ONE, HPNE, and HNE) were prepared in concentrations of 10 ng/μL using distilled diethyl ether. Each sample was introduced into the

mass spectrometer by a syringe pump (infusion rate of 20 μL/min) with the addition of hexane/2-propanol (197:3, v/v) at a flow rate of 0.5 mL/min.

Preparation of carboxynonanone-etheno-dGuo and [$^{15}N_5$]-analog

A solution of 13-HPODE (900 nmol) in ethanol (60 μL) and vitamin C (4.5 μmol) in Chelex-treated 100 mM MOPS containing 150 mM NaCl (pH 7.4, 10 μL) was added to dGuo (4.5 μmol) or [$^{15}N_5$]-dGuo (750 nmol) in Chelex-treated 100 mM MOPS containing 150 mM NaCl (pH 7.4, 1.1 mL). The reaction mixture was sonicated for 15 min at room temperature, incubated at 37°C for 24 h and then placed on ice. The samples were filtered through a 0.2-μm Costar cartridge prior to purification using gradient system 1. The [$^{15}N_5$]-carboxynonanone-etheno-dGuo was purified on a new column to prevent any contamination with the ^{14}N-analog. Concentrations were determined using UV spectrophotometery and assuming the same molecular extinction coefficient as heptanone-etheno-dGuo.[16]

Preparation of pure 15(*S*)-HPETE

Arachidonic acid (25 mg, 82.2 μmol) was added to soybean lipoxidase (type V, 1 mg) in 30 mL of 0.2 M Chelex-treated borate buffer (pH 9.0). The reaction was performed at 0°C under oxygen with constant stirring. After 4 h, the reaction mixture was acidified to pH 3 with 1 N HCl and the 15-HPETE was extracted with diethyl ether (2 × 5 mL). The combined extracts were washed with water, dried over sodium sulfate, and evaporated under a stream of nitrogen. The 15*S*-(*Z*,*Z*,*Z*,*E*)-HPETE was then purified using normal-phase gradient system 2 (retention time: 11.4 min). The pure 15*S*-(*Z*,*Z*,*Z*,*E*)-HPETE was dissolved in ethanol, and its concentration was determined by UV spectroscopy (λ_{max} 236 nm, $\varepsilon = 27\,000$). It was stored in ethanol at −70°C, and the solution was reanalyzed by reversed-phase LC/MS using gradient system 3 and MS operating conditions 1 before it was used. A single chromatographic peak was observed at a retention time of 9.5 min. The mass spectrum contained a dominant ammoniated molecule at *m*/*z* 354 $[M+NH_4]^+$ together with the expected fragment ion at *m*/*z* 219 $[M-C_6H_{12}OOH]^+$. When the pure 15*S*-(*Z*,*Z*,*Z*,*E*)-HPETE was analyzed by LC/MS/UV under normal-phase conditions using system 2 and MS operating conditions 1, no early eluting peaks corresponding to α,β-unsaturated aldehydes were detected.

Chelex-treated buffers

Reactions were conducted in transition metal ion free Chelex-treated buffer because it is known that all solvents contain transition metal ions.[27] Removal of the transition metal ions by Chelex was confirmed by incubation of the resulting buffer with vitamin C and showing that there was no decline in the absorbance at 265 nM.[27] This showed that there was <10 nM transition metal ions present.

Decomposition of 15(*S*)-HPETE

For quantitation experiments, 15(*S*)-HPETE (80 nmol, 400 μM) in Chelex-treated 100 mM MOPS buffer containing 150 mM NaCl (pH 7.0, 200 μL) was reacted with increasing amounts of vitamin C for 2 h at 37°C. Aldehydes were extracted into diethyl ether (200 μL), and an aliquot (50 μL) was analyzed by LC/APCI-MS/MS using gradient system 4 and operating conditions 2. Quantitation was performed from standard curves constructed by known amounts of authentic standards extracted from MOPS buffer with diethyl ether. Standards were made up at the following concentrations in diethyl ether: *t*-EDE and *c*-EDE (5 ng/μL), ONE (10 ng/μL), HPNE (10 ng/μL) and HNE (10 ng/μL).

Reaction of 15(*S*)-HPETE with dGuo in the presence of vitamin C

A solution of 15(*S*)-HPETE (150 nmol) in ethanol (10 μL) and vitamin C (750 nmol) in Chelex-treated 100 mM MOPS containing 150 mM NaCl (pH 7.4, 10 μL) was added to dGuo (750 nmol) in Chelex-treated 100 mM MOPS containing 150 mM NaCl (pH 7.4, 180 μL). The reaction mixture was sonicated for 15 min at room temperature, incubated at 37°C for 24 h, and then placed on ice. The samples were filtered through a 0.2-μm Costar cartridge prior to analysis of a portion of the sample (20 μL) by LC/MS using gradient system 5 and operating conditions 1.

Reaction of 15(*S*)-HPETE or 13(*S*)-HPODE with calf thymus DNA

15(*S*)-HPETE or 13(*S*)-HPODE (5 mM) in water was reacted with vitamin C (2 mM) in the presence of calf thymus DNA (5 mM) for 24 h. The reaction was placed on ice for 15 min. DNA was precipitated by cold ethanol and 2.9 M sodium acetate. The DNA was dissolved in 10 mM MOPS containing 100 mM NaCl (pH 7.0, 1 mL). DNAse I (556 units) dissolved in 10 mM MOPS containing 120 mM $MgCl_2$ (pH 7.0) was added and incubated at 37° for 1.5 h. Nuclease P1 was added along with 25 mM $ZnCl_2$ and incubated at 37°C for 2 h. Alkaline phosphatase was then added along with SAP buffer and incubated at 37°C for 1 h. The samples were filtered through a 0.2-μm Costar cartridge. At this time an aliquot was removed for LC/UV analysis of the bases using gradient 6. Quantitation of DNA bases was carried out by constructing standard curves of known amount of bases. The remaining sample was applied to a solid phase extraction (SPE) cartridge (6 mL, Supelclean LC-18; Supelco, Bellefonte, PA, USA) that had been pre-washed with acetonitrile (18 mL) and water (18 mL). The cartridge was then washed with water (4 mL) and methanol/water (1 mL; 5:95, v/v). Adducts were eluted with acetonitrile/water (6 mL; 1:1, v/v). The eluates were evaporated to dryness under nitrogen and dissolved in acetonitrile (160 μL), DIPE/acetonitrile (40 μL; 10:90, v/v), and PFB-Br/acetonitrile (100 μL; 20:80, v/v). The solution was left at room temperature for 1.5 h. It was then evaporated to dryness under nitrogen and dissolved in methanol/dichloromethane (100 μL; 25:75, v/v) and loaded onto a SPE cartridge (6 mL, Supelclean Si; Supelco) that had been pre-washed with dichloromethane (12 mL). The column was then washed with methanol/dichloromethane (3 mL; 1:99, v/v) and methanol/dichloromethane (1 mL; 5:95, v/v). Adducts were eluted with methanol/dichloromethane (6 mL; 25:75, v/v) and evaporated to dryness under nitrogen. Finally, the adducts were re-dissolved in acetonitrile/water (100 μL; 80:20, v/v). Aliquots (20 μL)

were analyzed by LC/APCI-MS/MS using gradient system 7 and operating systems 3. Internal standards of each adduct were added to the samples during hydrolysis and derivatization to account for any loss. Quantitation was performed from standard curves constructed by the peak area ratio of known amounts of authentic standards and internal standard. Precursor ions were monitored for the protonated molecule derived from the PFB of the endogenous adduct and its [$^{15}N_5$]-internal standard. Product ions that were monitored corresponded to the loss of the 2′-deoxyribose moiety (BH_2^+) from the endogenous adduct and its [$^{15}N_5$]-internal standard. Adduct levels were normalized to the amount of DNA as detected by base analysis mentioned above.

RESULTS

MS analysis of *t*-EDE and *c*-EDE

The APCI-MS and MS/MS spectra of *t*-EDE and c-EDE were identical. Each MS spectrum exhibited an intense $[MH]^+$ ion at *m/z* 169 together with a fragment ion at *m/z* 151 corresponding to $[MH-H_2O]^+$ (Fig. 1(A)). MS/MS analysis of *m/z* 169 gave rise to the following product ions at: *m/z* 151, $[MH-H_2O]^+$; *m/z* 133, $[MH-2H_2O]^+$; *m/z* 123, $[MH-H_2O-CO]^+$; *m/z* 113, $[MH-C_4H_8]^+$; *m/z* 109, $[MH-H_2O-C_3H_6]^+$; *m/z* 99, $[MH-C_5H_{10}]^+$; *m/z* 95, $[MH-H_2O-C_4H_8]^+$; *m/z* 85, $[MH-CO-C_4H_8]^+$; *m/z* 81, $[MH-H_2O-CO-C_3H_6]^+$; *m/z* 67, $[MH-H_2O-CO-C_4H_8]^+$ (Fig. 2(A)).

MS analysis of ONE

The APCI-MS spectrum of ONE showed an intense protonated molecule $[MH]^+$ at *m/z* 155 together with ions corresponding to $[MH-H_2O]^+$ at *m/z* 137 and the $[MH-H_2O-CO]^+$ ion at *m/z* 109 (Fig. 1(B)). MS/MS analysis of *m/z* 155 resulted in the formation of the following product ions at: *m/z* 137, $[MH-H_2O]^+$; *m/z* 127, $[MH-CO]^+$; *m/z* 119, $[MH-2H_2O]^+$; *m/z* 109, $[MH-H_2O-CO]^+$; *m/z* 95, $[MH-H_2O-C_3H_6]^+$; *m/z* 85, $[MH-CO-C_3H_6]^+$; *m/z* 67, $[MH-H_2O-CO-C_3H_6]^+$ (Fig. 2(B)).

MS analysis of HPNE

The APCI-MS spectrum of HPNE exhibited an intense $[MH-OH]^+$ ion at *m/z* 156 together with ions corresponding to $[MH]^+$ *m/z* 173, $[MH-HOOH]^+$ at *m/z* 139, and $[MH-2H_2O-CO]^+$ at *m/z* 109 (Fig. 1(C)). MS/MS analysis of *m/z* 156 resulted in the exclusive formation of the $[MH-OH-C_4H_9]^+$ ion at *m/z* 99 together with the following product ions at: *m/z* 137, $[MH-2H_2O]^+$; *m/z* 113, $[MH-OH-C_3H_7]^+$; *m/z* 109, $[MH-2H_2O-CO]^+$; *m/z* 95, $[MH-OH-H_2O-C_3H_7]^+$; *m/z* 67, $[MH-OH-H_2O-CO-C_3H_7]^+$ (Fig. 2(C)).

MS analysis of HNE

The APCI-MS spectrum of HNE showed an intense protonated molecule $[MH]^+$ at *m/z* 157 together with a $[MH-H_2O]^+$ ion at *m/z* 139 (Fig. 1(D)). MS/MS analysis of *m/z* 157 resulted in the formation of following product ions at: *m/z* 139, $[MH-H_2O]^+$; *m/z* 121, $[MH-2H_2O]^+$; *m/z* 111, $[MH-H_2O-CO]^+$; *m/z* 97, $[MH-H_2O-C_3H_6]^+$; *m/z* 81, $[MH-2OH-C_3H_6]^+$; *m/z* 69, $[MH-H_2O-CO-C_3H_6]^+$ (Fig. 2(D)).

Vitamin C mediated decomposition of 15(*S*)-HPETE

15(*S*)-HPETE was treated with increasing amounts of vitamin C and the products were identified by LC/APCI-MS based on their mass spectral characteristics and their LC retention times; as compared with standards, the products were found to *t*-EDE and *c*-EDE, ONE, HPNE, and HNE (data not shown). The products were then quantified by normal-phase LC/APCI-MS/MS. Representative chromatograms for the low dose vitamin C (100 μM; Fig. 4) and high dose vitamin C (2 mM; Fig. 5) are shown. *t*-EDE eluted at 10.4 min (*m/z* 169 → 99), *c*-EDE eluted at 11.4 min (*m/z* 169 → 99), ONE eluted at 10.8 min (*m/z* 155 → 95), HPNE eluted at 12.4 min

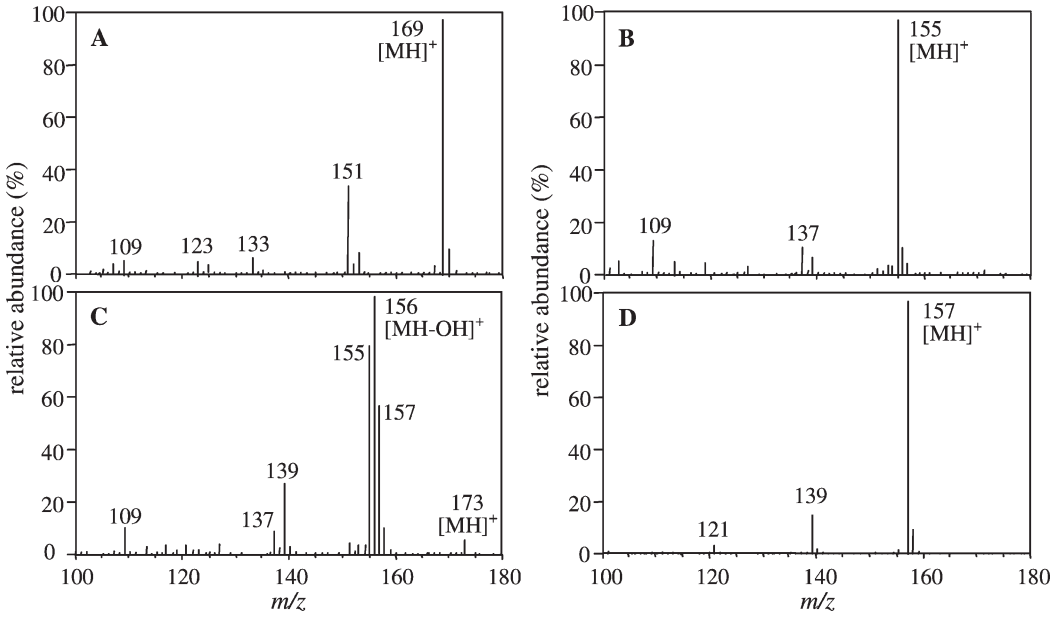

Figure 1. APCI-MS full scan spectra of authentic bifunctional electrophiles. (A) *t*-EDE and *c*-EDE; (B) ONE; (C) HPNE; and (D) HNE.

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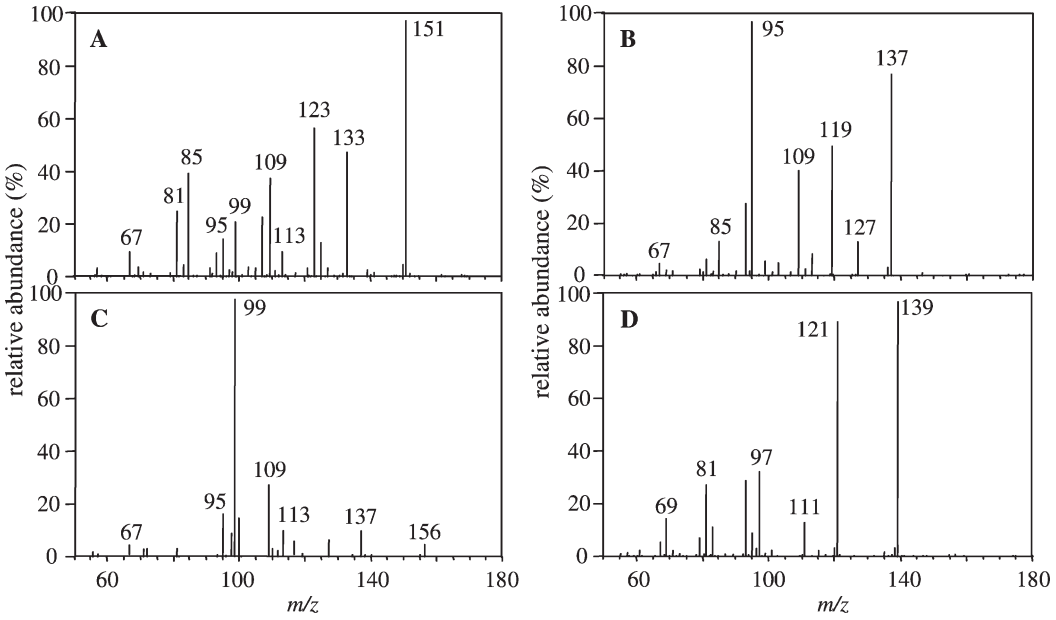


Figure 2. APCI-MS/MS product ion spectra for selected ions of authentic bifunctional electrophiles. (A) *t*-EDE and *c*-EDE (*m/z* 169→); (B) ONE (*m/z* 155→); (C) HPNE (*m/z* 156→); and (D) HNE (*m/z* 157→).

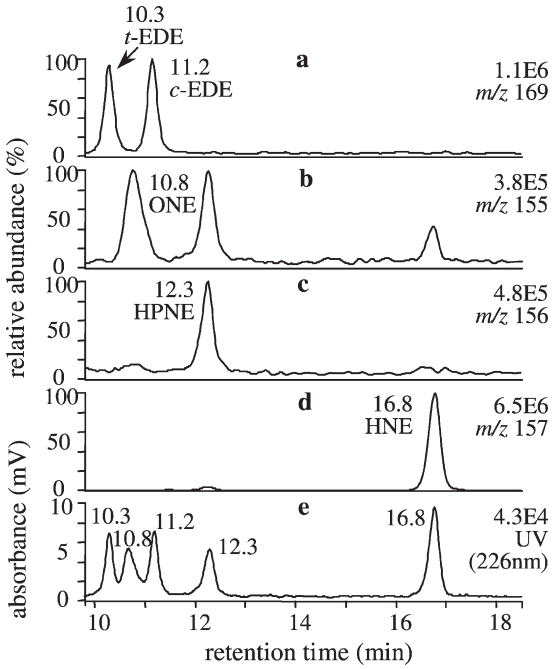


Figure 3. LC/APCI-MS/UV analysis of authentic bifunctional electrophile standards mixture: *t*-EDE (0.6 nmol), ONE (1.3 nmol), *c*-EDE (0.6 nmol), HPNE (1.2 nmol) and HNE (1.3 nmol). (a) Ion chromatograms (*m/z* 169) for the protonated molecule $[MH]^+$ of *t*-EDE and *c*-EDE. (b) Ion chromatogram (*m/z* 155) for $[MH]^+$ of ONE. (c) Ion chromatogram (*m/z* 156) for $[MH-OH]^+$ of HPNE. (d) Ion chromatogram (*m/z* 157) for $[MH]^+$ of HNE. (e) UV chromatogram monitoring at 226 nm.

(*m/z* 156 → 99), and HNE eluted at 16.8 min (*m/z* 157 → 139). At low vitamin C concentrations, the major products were *t*-EDE, HPNE, and ONE. As the vitamin C concentration increased, HPNE amounts decreased, with a concomitant increase in *t*-EDE, *c*-EDE, ONE, and HNE (Fig. 5).

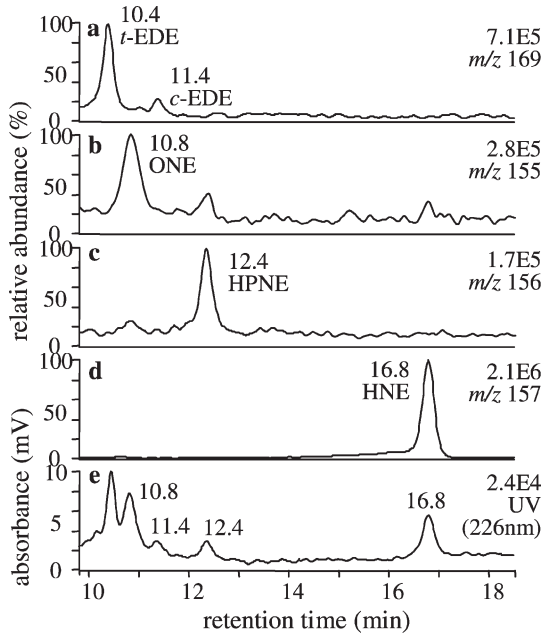


Figure 4. LC/APCI-MS/UV analysis of reaction between 15-HPETE (371 μM) and vitamin C (100 μM) in transition metal ion free MOPS buffer (100 mM) containing NaCl (150 mM) at pH 7.0 and 37°C for 2 h. Ion (a–d) and UV (e) chromatograms are the same as in Fig. 3.

Vitamin C mediated decomposition of 15(*S*)-HPETE in the presence of dGuo

LC/MS analysis of the reaction mixture revealed two major adducts. The mass spectrum of the most polar adduct (10.4 min) revealed an intense protonated molecule $[MH]^+$ at *m/z* 292, together with a BH_2^+ ion at *m/z* 176. MS^2 analysis of MH^+ resulted in a BH_2^+ product ion. These LC/MS characteristics were identical to those for 1,N^2-etheno-dGuo (E-dGuo). LC/MS analysis of the most abundant adduct

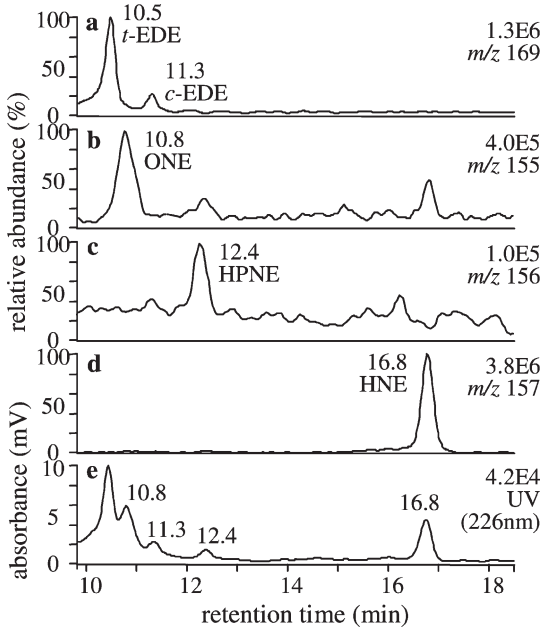


Figure 5. LC/APCI-MS/UV analysis of reaction between 15-HPETE (371 μM) and vitamin C (2 mM) in transition metal ion free MOPS buffer (100 mM) containing NaCl (150 mM) at pH 7.0 and 37°C for 2 h. Ion (a–d) and UV (e) chromatograms are the same as in Fig. 3.

(20.6 min) showed an MH^+ at *m/z* 404, together with BH_2^+ at *m/z* 288. MS^2 analysis of MH^+ gave rise to an intense BH_2^+ ion. Further CID of the BH_2^+ ion at *m/z* 288 (MS^3) gave rise to ions at *m/z* 190 ($BH_2^+ - C_5H_{11}CO$) and 260 ($BH_2^+ - CO$). These mass spectral characteristics were consistent with heptanone etheno-dGuo (heptanone-etheno-dGuo). The small peak (19.6 min) that eluted prior to heptanone-etheno-dGuo was identified as heptanone-ethano-dGuo (MH^+; *m/z* 422, BH_2^+; *m/z* 306). (Fig. 7).

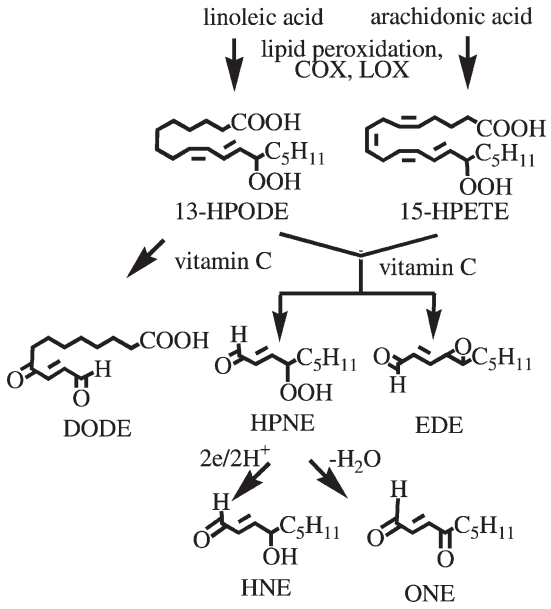
Vitamin C mediated decomposition of 13-HPODE and 15-HPETE with calf-thymus DNA

15(*S*)-HPETE or 13(*S*)-HPODE and vitamin C was decomposed in the presence of varying amounts of calf thymus DNA. It was found that a 1:1 concentration of lipid hydroperoxide to DNA yielded the best results (data not shown). LC/APCI-MS/MS in the multiple-reaction monitoring (MRM) mode was conducted to provide maximal sensitivity and specificity for the quantification of the dGuo adducts formed. Representative chromatograms for the 13(*S*)-HPODE- (Fig. 8) and 15(*S*)-HPETE- (Fig. 9) derived DNA adducts and their internal standards are shown. Etheno-dGuo (*m/z* 472 → 356) and [$^{15}N_5$]-etheno-dGuo (*m/z* 477 → 361) eluted at 22.4 min, heptanone-etheno-dGuo (*m/z* 584 → 468) and [$^{15}N_5$]-heptanone-etheno-dGuo (*m/z* 589 → 473) eluted at 27.9 min, and carboxynonanone-etheno-dGuo (*m/z* 836 → 522) and [$^{15}N_5$]-carboxynonanone-etheno-dGuo (*m/z* 841 → 527) eluted at 30.6 min. Both 13(*S*)-HPODE and 15(S)-HPETE formed heptanone-1,N^2-etheno-dGuo (42 adducts/10^6 bases compared with 33 adducts/10^6 normal bases) and 1,N^2-etheno-dGuo (each gave approximately 4 adducts/10^6 normal bases); however, only 13(*S*)-HPODE produced carboxynonanone-etheno-dGuo (over 92 adducts/10^6 normal bases) (Fig. 10).

DISCUSSION

In a previous study we showed that vitamin C mediated decomposition of the linoleate-derived lipid hydroperoxide 13-HPODE results in the formation of the α,β-unsaturated aldehydes, HPNE, EDE, ONE and HNE.[11] We also demonstrated that HPNE was the precursor to the formation of ONE and HNE.[11] HNE has also been shown to arise from HPNE during the autoxidation of 13-HPODE (Scheme 1).[12] Studies of 13-HPODE decomposition and the analysis of DNA and protein adducts that can be formed have relied heavily on the use of LC/electrospray ionization (ESI)-MS methodology.[16–19,28–30] Three of the bifunctional electrophiles that arise from 13-HPODE decomposition (*t*-EDE, *c*-EDE, and ONE) are poorly ionized under ESI conditions. In order to analyze the products of vitamin C mediated 13-HPODE decomposition, it was necessary to use LC/APCI-MS in combination with normal-phase chromatography.[4] We have now used this methodology to determine whether vitamin C mediated decomposition of 15(*S*)-HPETE results in formation of the same bifunctional electrophiles that were detected from the decomposition of 13-HPODE (Scheme 1). Analysis of the individual synthetic bifunctional electrophiles revealed MH^+ for *t*-EDE, *c*-EDE, ONE and HNE at *m/z* 169 (Fig. 1(A)), 169 (Fig. 1(A)), 155 (Fig. 1(B)), and 157 (Fig. 1(D)), respectively. The MH^+ for HPNE at *m/z* 173 was very weak with MH^+-OH at *m/z* 156 as the major ion in the APCI mass spectrum (Fig. 1(C)). The loss of OH is thought to occur in the APCI source. The neutral radical that is produced and any undecomposed parent are then both protonated. The ratio between $[MH]^+$ and $[MH\text{-}OH]^+$ may reflect the pre-ionization ratio of the two species. This is consistent with the observation that the 17 Da loss is a minor process observed in the CID product ion spectrum, although an ion arising from the loss of 17 Da is the base peak in the unimolecular spectrum. Product ion spectra of individual bifunctional electrophiles (Fig. 2) were used to confirm their identity during LC/MS analysis. The bifunctional electrophiles were all separated under normal phase LC conditions (Fig. 3). Quantitation was performed using selected-ion monitoring (SIM) analysis of the most intense ions present in the APCI mass spectra (Fig. 3). HNE was more readily ionized than the other bifunctional electrophiles so that its APCI response was almost an order of magnitude more intense (Fig. 3(d)).

15(*S*)-HPETE was treated with vitamin C (100 μM) for 2 h at 37°C and the products were analyzed by LC/APCI-MS. HPNE, *t*-EDE, and ONE were the major products of the reaction as evidenced by the APCI-MS response (Figs. 4(a)–4(c)) and the LC/UV response (Fig. 4(e)). Minor amounts of HNE (Figs. 4(d) and 4(e)) and *c*-EDE were produced (Figs. 4(a) and 4(e)) (Scheme 2). When the concentration of vitamin C was increased to 2 mM, the amount of HPNE was decreased markedly (Figs. 5(c) and 5(e)). There was a concomitant increase in the amount of *t*-EDE and ONE (Figs. 5(a), 5(b),



Scheme 2. Vitamin C mediated decomposition of linoleic acid and arachidonic acid derived lipid hydroperoxides to bifunctional electrophiles.

and 5(e)). Concentrations of bifunctional electrophiles formed from 15(*S*)-HPETE over a range of vitamin C concentrations were determined by normal-phase LC/APCI-MS (Fig. 6). In contrast to a previous study with 13-HPODE,[11] the concentration of *t*-EDE was always greater than that of ONE (Fig. 6). At concentrations >250 μM vitamin C there was a decline in HPNE with a concomitant increase in the amounts of ONE and HNE (Fig. 6). This is directly analogous to what was observed with 13-HPODE and is due to the conversion of HPNE into ONE and HNE.[11]

When homolytic decomposition of 15(*S*)-HPETE was initiated by vitamin C in the presence of dGuo, the formation of etheno-dGuo and heptanone-etheno-dGuo adducts was observed by LC/APCI-MS (Fig. 7). There was a marked increase in the amount of the unsubstituted etheno-dGuo

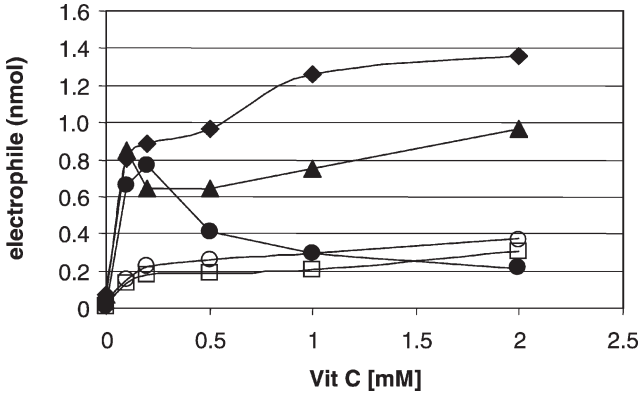


Figure 6. Amount of bifunctional electrophiles formed over a range of vitamin C concentrations (0.1–2.0 mM). Determinations were conducted in duplicate. 4-ONE, solid triangles; *t*-4,5-EDE, solid diamonds; *c*-4,5-EDE, open squares; 4-HPNE, solid circles; 4-HNE, open circles.

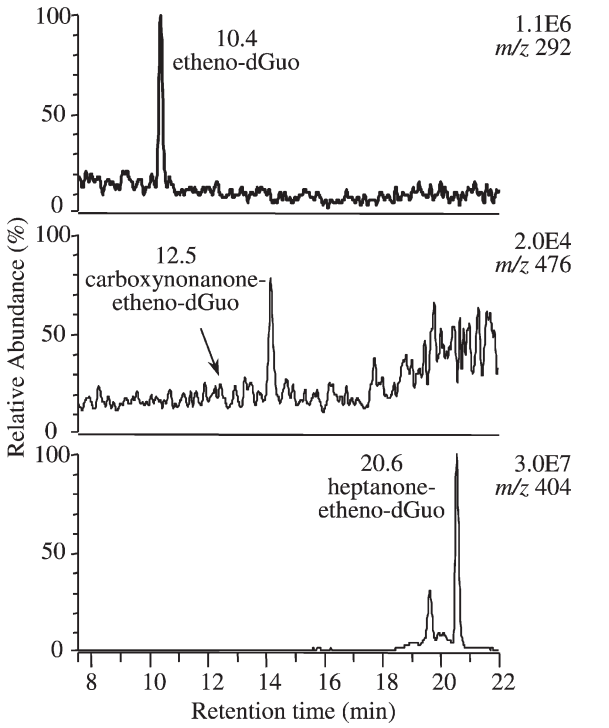


Figure 7. LC/APCI-MS/MS chromatograms for the decomposition of 15(*S*)-HPETE by vitamin C in the presence of dGuo.

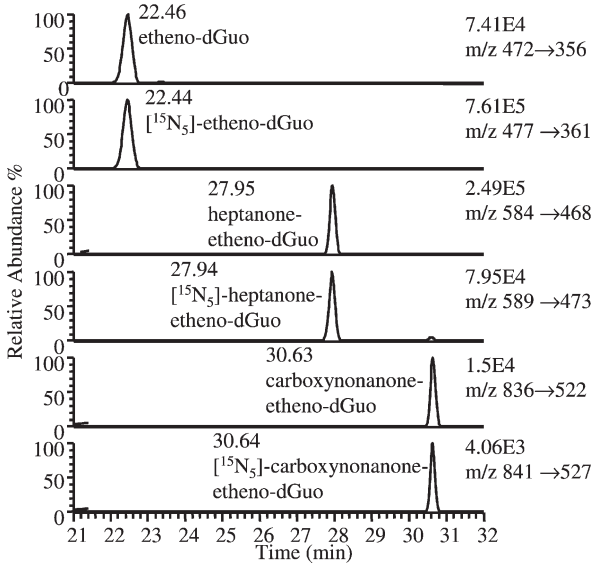


Figure 8. LC/APCI-MS/MS analysis of DNA adducts from the reaction of 13(*S*)-HPODE in the presence of vitamin C and calf thymus DNA. Ion chromatograms for etheno-dGuo (*m/z* 472 → 356), [$^{15}N_5$]-etheno-dGuo (*m/z* 477 → 361), heptanone-etheno-dGuo (*m/z* 584 → 468), [$^{15}N_5$]-heptanone-etheno-dGuo (*m/z* 589 → 473), carboxynonanone-etheno-dGuo (*m/z* 836 → 522), and [$^{15}N_5$]-carboxynonanone-etheno-dGuo (*m/z* 841 → 527).

adduct when compared with the reaction between 13(*S*)-HPODE and dGuo (data not shown). Furthermore, none of the DODE-derived carboxynonanone-etheno-dGuo adduct was detected (Fig. 7). This finding lends credence to the

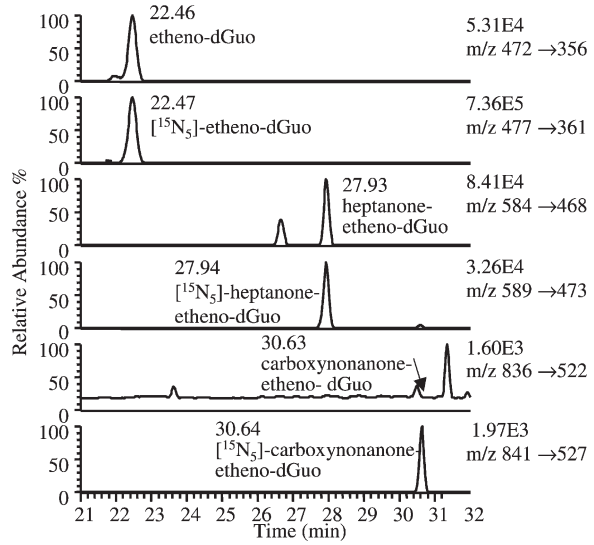


Figure 9. LC/APCI-MS/MS analysis of DNA adducts from the reaction of 15(*S*)-HPETE in the presence of vitamin C and calf thymus DNA. Ion chromatograms for etheno-dGuo (*m/z* 472 → 356), [$^{15}N_5$]-etheno-dGuo (*m/z* 477 → 361), heptanone-etheno-dGuo (*m/z* 584 → 468), [$^{15}N_5$]-heptanone-etheno-dGuo (*m/z* 589 → 473), carboxynonanone-etheno-dGuo (*m/z* 836 → 522), and [$^{15}N_5$]-carboxynonanone-etheno-dGuo (*m/z* 841 → 527).

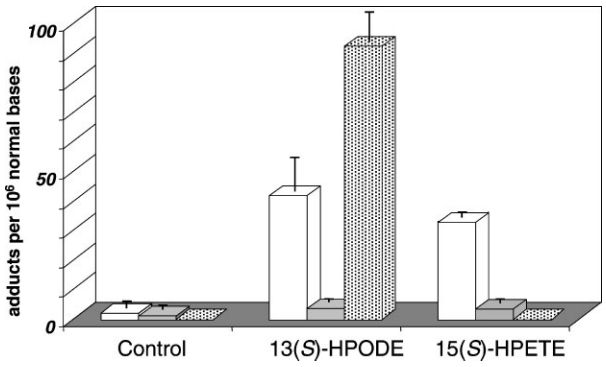


Figure 10. Amount of DNA adducts (adducts/10^6 normal bases) formed from the decomposition of 13(*S*)-HPODE or 15(*S*)-HPETE. Determinations were conducted in triplicate (means ± SEM). Heptanone-etheno-dGuo, white bars; etheno-dGuo, solid grey bars; carboxynonanone-etheno-dGuo, dotted bars.

proposed pathway for DODE formation. It has been suggested that DODE arises from a Hock rearrangement of a *bis*-hydroperoxide intermediate formed during 13-HPODE decomposition.[15] A similar pathway of 15-HPETE decomposition would not result in the formation of DODE.

A quantitative comparison of 15(*S*)-HPETE- and 13(*S*)-HPODE-mediated etheno-dGuo-adduct formation in calf thymus DNA was made. The DNA was hydrolyzed and DNA adducts were converted into PFB derivatives prior to LC/APCI-MRM/MS analysis of the PFB derivatives. This simple derivatization procedure provided a significant increase in sensitivity when compared with analysis of the underivatized analytes under both ESI and APCI conditions. This was particularly relevant to the carboxynonanone-etheno-dGuo, which was very poorly ionized before derivatization. The ready loss of 116 Da from all adducts during CID, corresponding to a 2′-deoxyribose moiety, is typical of DNA bases. The resulting BH_2^+ ions, the use of carefully controlled experiments, and stringent HPLC conditions provided high sensitivity and specificity when MRM profiling was conducted. Synthetic [$^{15}N_5$]-analogs were used as internal standards and quantitation was performed using standard stable isotope dilution methodology.

There was no quantitative difference in the formation of etheno-dGuo between the two lipid hydroperoxides (Fig. 10). A previous study has shown that *t*-EDE is a precursor in the formation of etheno-dGuo.[9] The increased *t*-EDE formation in the reactions between 15(*S*)-HPETE and vitamin C (Fig. 6) when compared with 13(*S*)-HPODE suggested that there would be a concomitant increase in etheno-dGuo formation.[11] The lack of any such increase (Fig. 10) suggests that there must be an alternative pathway for the formation of unsubstituted etheno-dGuo adducts. However, the decreased amount of heptanone-etheno-dGuo adduct formation (Fig. 10) from 15(*S*)-HPETE when compared with the 13(*S*)-HPODE reaction (Figs. 9 and 10) was consistent with the reduced amount of ONE formation (Fig. 6) from 15(*S*)-HPETE in comparison with 13(*S*)-HPODE.[11] No carboxynonanone-etheno-dGuo was detected in the reaction of 15(*S*)-HPETE with calf thymus DNA, whereas carboxynonanone-etheno-dGuo was the major adduct in the 13(*S*)-HPODE-mediated pathway (Fig. 10). This is in keeping with the proposed pathway for decomposition of 13(*S*)-HPODE to DODE.[15] Therefore, these studies raise the possibility that carboxynonanone-etheno-dGuo could be a specific marker of linoleic acid derived DNA damage. A similar concept has emerged in the studies of leukotriene (LT) biosynthesis. A carboxylate-containing DNA adduct, which arises from the reaction between LTA_4 and DNA, has been characterized and an LC/MS assay has been developed for its quantiation.[31,32]

In summary, LC/MS-based methodology revealed that *t*-EDE was the major bifunctional electrophile formed during vitamin C mediated 15(*S*)-HPETE decomposition. Stable isotope dilution LC/MS studies showed that this resulted in no increased levels of unsubstituted etheno-dGuo adducts when compared with 13(*S*)-HPODE. However, the formation of heptanone-etheno-dGuo adducts in calf thymus DNA was reduced when compared with 13(*S*)-HPODE. In contrast to reactions conducted using 13(*S*)-HPODE, no carboxy-containing adducts were observed. The ability to monitor both chiral lipid biosynthesis[24] and to quantify the resulting DNA adducts will make it possible to study the role of COXs and LOXs as mediators of DNA-adduct formation in cells and *in vivo* in human and animal models. Such studies are now in progress.

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