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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF LINOLEIC ACID HYDROPEROXIDES AND THEIR CORRESPONDING ALCOHOL DERIVATIVES

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## SUMMARY

The four major hydroperoxides derived from autoxidation or lipoxygenase action on linoleic acid or on methyl linoleate and their corresponding alcohol derivatives are resolved by high-performance liquid chromatography on Zorbax SIL 3 μm particulate columns irrigated with hexane–diethyl ether–acetic acid mixtures. The four major linoleic acid hydroperoxides are interconverted at different rates in benzene or carbon tetrachloride solutions but are stable to storage under nitrogen at −70°C for several months.

## INTRODUCTION

The oxidation of polyunsaturated fatty acids by lipoxygenases and by autoxidation yields isomeric fatty acyl hydroperoxides, and subsequent transformations may yield the corresponding alcohols. Resultant hydroperoxide mixtures have been analyzed as methyl esters or as the reduced alcohol methyl esters by high-performance liquid chromatography (HPLC), but much less attention has been given to HPLC systems resolving underivatized mixtures of hydroperoxides and corresponding alcohols. For linoleic acid hydroperoxides HPLC on silica or polymer gel with binary solvent mixtures of hexane and ethanol or with ternary mixtures of hydrocarbon, alcohol, and acetic acid have been used but with inadequate resolution of the four isomeric hydroperoxides 9-hydroperoxy-(10*E*,12*Z*)-octadeca-10,12-dienoic (abbreviated 9EZ-OOH), 9-hydroperoxy-(10*E*,12*E*)-octadeca-10,12-dienoic (9EE-OOH), 13-hydroperoxy-(9*Z*,11*E*)-octadeca-9,11-dienoic (13ZE-OOH), and 13-hydroperoxy-(9*E*,11*E*)-octadeca-9,11-dienoic (13EE-OOH) acids generally encountered[1–6]. However, a binary mixture of heptane and acetic acid does resolve the four regioisomers[7].

In seeking to devise HPLC systems that resolve both the four isomeric linoleic acid hydroperoxides and their corresponding alcohol derivatives we examined both adsorption and reversed phase HPLC systems and describe here several systems that are useful in the analysis of oxidized linoleic acid and for preparation of pure samples of individual isomeric hydroperoxides and alcohols.

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## EXPERIMENTAL

Linoleic acid, methyl linoleate, soybean lipoxygenase Type V, and peanut acetone powder were purchased from Sigma (St. Louis, MO, U.S.A.). Chromatography solvents were those of Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Solvents were filtered before use through Gelman Sciences (Ann Arbor, MI, U.S.A.) 0.45-$\mu$m membrane filters.

Linoleic acid and methyl linoleate hydroperoxides were obtained by autoxidation of 500 mg pure substrate in 25 ml dry benzene at room temperature by sparging the solution with oxygen. Solvent level was maintained at the same level during the oxidation. Oxidized material was subjected to thin-layer chromatography using silica gel PK6F and K6F chromatoplates (Whatman) irrigated with hexane–diethyl ether–acetic acid (70:30:1) for linoleic acid hydroperoxides, (80:20:1) for methyl linoleate hydroperoxides. Hydroperoxides were visualized with N,N-dimethyl-*p*-phenylenediamine spray[8]. Individual hydroperoxides were recovered from the chromoplates for further use. Reduction of the purified single isomeric hydroperoxides by $NaBH_4$ in methanol afforded the corresponding alcohol derivatives.

Solutions (20 $\mu$g/ml) of individual linoleic acid hydroperoxides in dry benzene or in carbon tetrachloride were held at room temperature or at $-70$°C and analyzed by HPLC periodically by direct injection of the solution onto the Zorbax SIL column.

Soybean lipoxygenase incubations were conducted by evaporating a solution of 250 mg linoleic acid in 25 $\mu$l 25% Tween-20 in acetone in a 125-ml flask, evaporating the solvent, dispersing the substrate in 50 ml oxygen saturated 0.1 *M* pH 9.2 borate buffer or 0.1 *M* pH 5.5 acetate buffer and adding 700 units of enzyme. Incubation flasks were shaken in a water bath (22°C) for 30 min, the preparation acidified to pH 2.0 and extracted three times with 50 ml diethyl ether. The ether extracts were evaporated under vacuum and product hydroperoxides resolved by thin-layer chromatography directly. The total yield of linoleic acid hydroperoxides was 14.4% at pH 9.2, 3.2% at pH 5.5.

Peanut lipoxygenase incubations were conducted with enzyme purified from peanut acetone powder. Acetone powder was stirred with 200 ml 0.1 *M* pH 7.0 Tris–HCl buffer at room temperature for 1 h, and the slurry was centrifuged at 12 000 *g* for 10 min. The pellet was redissolved in buffer and solid ammonium sulfate added. Material precipitating between 40% and 60% saturation was redissolved in 20 ml buffer containing 5 ml ethanol and held overnight at 4°C. After centrifugation for 20 min at 25 000 *g* the supernate was dialyzed against 2 l 0.1 *M* pH 7.0 phosphate buffer for 2 days. Solid ammonium sulfate was added to give 80% saturation, and the resultant precipitate was recovered by centrifugation at 30 000 *g* for 15 min. The pellet was dissolved in 10 ml pH 7.0 phosphate buffer and 0.5 ml taken for incubation with 250 mg linoleic acid in 0.1 *M* pH 9.2 borate buffer or 0.1 *M* pH 5.5 acetate buffer in the same manner used for soybean lipoxygenase incubations.

Chromatography was conducted with HPLC equipment of Waters Assoc. (Milford, MA, U.S.A.) using a 8 cm × 6.2 mm Zorbax SIL 3 $\mu$m particulate adsorption column (DuPont Instruments, Wilmington, DE, U.S.A.) with ternary solvent mixtures identified in Table I or with a $C_{18}$-type reversed-phase Waters Assoc. fatty acid analysis column with quaternary solvents (Table I). Effluent monitoring was by means of a Perkin-Elmer variable wavelength spectrophotometric detector

Model LC-55 set at 234 nm. Amounts of resolved components were estimated from measured areas under the appropriate peak on elution curves in comparison with those of graded amounts of pure analyte.

## RESULTS AND DISCUSSION

Resolution of the individual linoleic acid hydroperoxides was most simply accomplished using Zorbax SIL 3 $\mu$m particulate absorption columns with ternary solvent mixtures patterned after previously described systems. Thus, inclusion of small amounts of diethyl ether in the binary system heptane–acetic acid of Koskas *et al.*[7] gave improved resolution of the four hydroperoxides and led us to the derived solvent mixtures of hexane–diethyl ether–acetic acid, systems A–E of Table I. These systems listed in order of increasing ether content provide a series of increasing polarities useful for a variety of purposes.

The four hydroperoxides and their corresponding alcohols are resolved suitably in systems B or C from one another asnd from the four derived alcohols. Their methyl esters are also resolved by system A (Fig. 1). These systems permit analysis of oxidized linoleic acid mixtures (or their methyl esters) in one chromatogram. These new systems also reduce the amount of acetic acid used, thus avoiding some alteration of column effectiveness and permitting effluent monitoring at 234 nm as well.

Resolution of the hydroperoxides as a group was readily accomplished using system D, with the corresponding alcohol derivatives being resolved. The derived alcohols are eluted as a group by system E. Elution of the four hydroperoxides as a group and of the derived alcohols as a group was also obtained using the reversed phase column with system G. The two *cis,trans* (*ZE*) hydroperoxides were resolved from the *trans,trans* (*EE*) isomers as groups and from the derived four alcohols as a group by system F (Fig. 2).

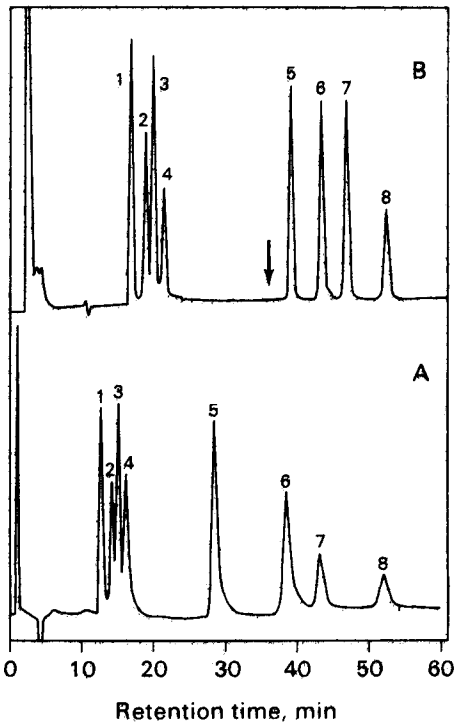
These systems permit considerable flexibility in analyzing complex mixtures of

TABLE I

CHROMATOGRAPHY SYSTEMS FOR ANALYSIS OF OXIDIZED LINOLEIC ACID

| *System* | *Solvent composition* | *Usefulness** |
|---|---|---|
| *Zorbax SIL column with hexane–diethyl ether–acetic acid* | | |
| A | 1000:100:1 | Resolution of 18:2-OOH methyl esters at 1 ml/min; thereafter resolution of 18:2-OH methyl esters at 2 ml/min |
| B | 1000:150:1 | Resolution of 18:2-OOH at 2 ml/min, thereafter resolution of 18:2-OH at 3 ml/min |
| C | 1000:200:1 | Resolution of 18:2-OOH and 18:2-OH at 2 ml/min |
| D | 1000:300:1 | Resolution of 18:2-OOH as a group with resolution of 18:2-OH at 2 ml/min |
| E | 1000:1100:11 | Resolution of 18:2-OH as a group at 2 ml/min |
| *Fatty acid analysis column with tetrahydrofuran–acetonitrile–water–acetic acid* | | |
| F | 25:50:100:1 | Resolution of 18:2(*EE*)-OOH and 18:2(*EZ*)-OOH in groups and of 18:2-OH as a group at 1 ml/min |
| G | 50:80:100:1 | Resolution of 18:2-OOH and 18:2-OH as groups at 1 ml/min |

* Abbreviations: 18:2-OOH for linoleic acid hydroperoxide; 18:2-OH for corresponding hydroxyoctadecadienoic acids.



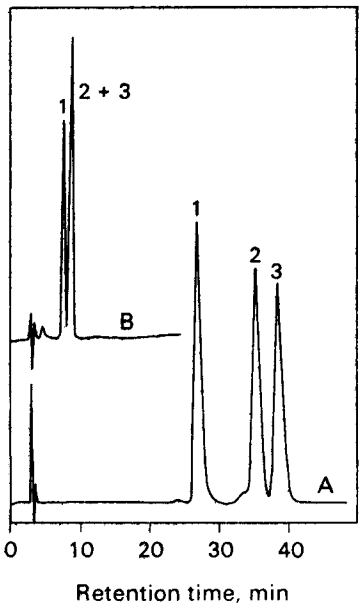




Fig. 1. High-performance liquid chromatograms on Zorbax SIL columns. (A), Linoleic acid hydroperoxides and corresponding alcohols in system C at 2 ml/min. (B) Methyl linoleate hydroperoxides and corresponding alcohols in system A at 1 ml/min to the arrow, 2 ml/min thereafter. Component identities: 1 = 13ZE-OOH; 2 = 13EE-OOH; 3 = 9EZ-OOH; 4 = 9EE-OOH; 5 = 13ZE-OH; 6 = 13EE-OH; 7 = 9EZ-OH; 8 = 9EE-OH.

Fig. 2. Reversed-phase high-performance liquid chromatography of linoleic acid hydroperoxides and their corresponding alcohols. (A) System F at 1 ml/min; (B) system G at 1 ml/min. Component identities: 1 = regioisomeric hydroxyoctadecadienoic acids 13ZE-OH, 13EE-OH, 9EZ-OH, and 9EE-OH; 2 = *cis,trans*-hydroperoxides 13ZE-OOH and 9EZ-OOH; 3 = *trans,trans*-hydroperoxides 13EE-OOH and 9EE-OOH.

these eight components and in preparing individual components or groups of components for other work. In several of these systems where both hydroperoxides and derived alcohols are to be analyzed, flow-rates may be adjusted following resolution of the more mobile hydroperoxides, thereby to hasten resolution of the more polar alcohols.

System A is useful for the resolution of methyl linoleate hydroperoxides and derived alcohols, whereas prior hexane–ethanol binary mixtures often used do not accord this result. The hexane–ethanol system of Chan and Levett[9], for instance, in our hands eluted the most mobile 13ZE-OOH methyl ester and its derived alcohol in that sequence, followed by the next most mobile 13EE-OOH methyl ester and its correspoonding alcohol. The isomeric 9-hydroperoxide methyl esters were also eluted in that order but with the 9EZ-OH and 9EE-OOH methyl esters unresolved from one another. This elution order precludes ready elution of groups of hydroperoxides or alcohols, and the failure to resolve a methyl ester hydroperoxide from a methyl

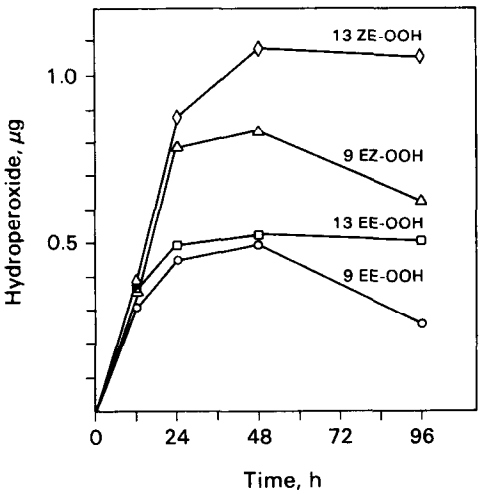



Fig. 3. High-performance liquid chromatographic analysis of linoleic acid hydroperoxides formed in benzene solution sparged with oxygen.

ester alcohol of another configuration limits the usefulness of such system where these items are in importance.

These systems were also suitable for estimation of amounts of resolved components by simple peak area measurements, as a good linearity of response was had for the hydroperoxides over the range 0–1.7 μg, for the corresponding alcohols over the range 0–7 μg.

The utility of these improved systems was evaluated by studying the autoxidation of linoleic acid in benzene solution, the peroxidation of linoleic acid by soybean and peanut lipoxygenases in buffered media, and the interconversions of the four linoleic acid hydroperoxides and alcohols in benzene and in carbon tetrachloride.

The autoxidation of linoleic acid in benzene solution produced all four regioisomeric hydroperoxides early in the reaction, but only the 13ZE-OOH and 13EE-OOH isomers maintained their levels, the 9EZ-OOH and 9EE-OOH isomers declining in amounts, *cf.* Fig. 3. These losses appear to be from hydroperoxide isomerizations and interconversions but also from hydroperoxide decompositions to products not apparent on the chromatograms.

The two *trans,trans* hydroperoxides were more stable than their *cis,trans* isomers, 13EE-OOH and 9EE-OOH being equally stable up to 4 days in benzene, with a sharp decline in stability thereafter. In carbon tetrachloride 13EE-OOH was more stable than 9EE-OOH. However, both *trans,trans* hydroperoxides underwent the same isomerizations, 13EE-OOH and 9EE-OOH being interconverted in both benzene and carbon tetrachloride as the major process, with 13EE-OOH isomerizing to 13ZE-OOH and 9EE-OOH to 9EZ-OOH as slower processes.

The *cis,trans* hydroperoxides were less stable and presented a more complex set of reactions. The 13ZE-OOH was transformed to its more stable 13EE-OOH isomer as a major process, with the 9EE-OOH also formed. The 9EZ-OOH was the least stable of all, being transformed rapidly and completely equally to the 13ZE-

OOH, 13EE-OOH, and 9EE-OOH in benzene, to 9EE-OOH with small amounts of the others in carbon tetrachloride.

Thus, five processes occur in this system: (i) interconversion of the *trans,trans* isomers 13EE-OOH and 9EE-OOH, (ii) rearrangement of the *cis,trans* diene to *trans,trans* diene (13ZE-OOH to 13EE-OOH, 9EZ-OOH to 9EE-OOH), (iii) isomerization of *cis,trans* hydroperoxide to *trans,trans* isomeric hydroperoxide (13ZE-OOH to 9EE-OOH, 9EZ-OOH to 13EE-OOH), (iv) *trans,trans* diene rearrangement to *cis,trans* diene (9EE-OOH to 9EZ-OOH, 13EE-OOH to 13ZE-OOH), and (v) rearrangement of the least stable *cis,trans* hydroperoxide to the more stable *cis,trans* isomer (9EZ-OOH to 13EZ-OOH).

Only traces of derived alcohols were detected in the transformations of 13ZE-OOH in benzene. In the other cases the corresponding alcohols were not detected. However, the 9EZ-OOH in carbon tetrachloride formed small amounts of an unidentified product with mobility intermediate between that of the hydroperoxides and that of the derived alcohols. The derived alcohols also appear to be subject to isomerizations but are also thermally decomposed extensively to undetectable products (Table II).

The rearrangement of individual methyl linoleate hydroperoxide isomers to mixtures of all four possible isomers has been previously described[10,11] and the isomerization of linoleic acid 13-hydroperoxides has been examined[5,11]. However, our present data describe the process for linoleic acid hydroperoxides in greater detail than heretofore.

TABLE II

STABILITIES OF LINOLEIC ACID HYDROPEROXIDES AND ALCOHOLS

| *Isomer added* | *Isomer found* | *Hydroperoxide (%)* | | | | *Alcohol (%)* | |
|---|---|---|---|---|---|---|---|
| | | *Benzene* | | *Carbon tetrachloride* | | *Benzene 96 h* | *Carbon tetrachloride 96 h* |
| | | *48 h* | *96 h* | *48 h* | *96 h* | | |
| 13ZE | 13ZE | 51.0 | 30.9 | 16.8 | 4.6 | 50.3 | 47.8 |
| | 13EE | 7.0 | 17.0 | 14.7 | 17.0 | 0 | 2.5 |
| | 9EZ | 0.5 | 0 | 0 | 0 | 0 | 0 |
| | 9EE | 9.3 | 7.7 | 12.4 | 11.6 | 0 | 0 |
| 13EE | 13ZE | 0 | 4.2 | 1.9 | 4.2 | 0 | 0 |
| | 13EE | 88.8 | 25.1 | 38.6 | 26.9 | 10.3 | 9.5 |
| | 9EZ | 0 | 0.9 | 0 | 0 | 0 | 0 |
| | 9EE | 1.4 | 16.7 | 16.7 | 24.2 | 0 | 0 |
| 9EZ | 13ZE | 13.7 | 9.2 | 2.3 | 0 | 0 | 0 |
| | 13EE | 9.2 | 6.5 | 18.7 | 0.8 | 0 | 0 |
| | 9EZ | 19.1 | 6.5 | 0 | 0 | 33.1 | 21.1 |
| | 9EE | 8.4 | 8.0 | 26.0 | 0.8 | 0 | 2.2 |
| 9EE | 13ZE | 0.9 | 9.4 | 6.4 | 3.8 | 0 | 0 |
| | 13EE | 7.7 | 9.4 | 29.0 | 10.7 | 0 | 0 |
| | 9EZ | trace | 5.8 | 2.6 | 0 | 0 | 0 |
| | 9EE | 88.9 | 15.4 | 20.1 | 7.7 | 39.3 | 21.7 |

TABLE III

LIPOXYGENASE ACTION ON LINOLEIC ACID

| *Product formed* | *Amount formed by soybean lipoxygenase (%)** | | *Amount formed by peanut lipoxygenase (%)** | |
|---|---|---|---|---|
| | *pH 5.5* | *pH 9.2* | *pH 5.5* | *pH 9.2* |
| 13ZE-OOH | 71.4 (62.2) | 98.0 (85.1) | 50.2 (41.3) | 81.0 (66.5) |
| 13EE-OOH | 7.1 (11.1) | 0.5 (2.9) | 11.0 (15.0) | 0 (5.2) |
| 9EZ-OOH | 9.7 (13.1) | 1.2 (9.5) | 37.4 (32.6) | 19.0 (23.8) |
| 9EE-OOH | 11.7 (13.5) | 0.3 (2.5) | 1.4 (11.1) | 0 (4.5) |

* Data in parentheses are total amounts formed by lipoxygenase action and concommitant autoxidation.

These hydroperoxide decompositions were retarded by storage of the benzene or carbon tetrachloride solutions under nitrogen at −70°C. After two months of such storage, the 13ZE-OOH had isomerized to 1.5% 13EE-OOH and 1.0% 9EZ-OOH in benzene, to 0.5% 13EE-OOH and 1.0% 9EZ-OOH in carbon tetrachloride.

The utility of the improved HPLC system was further demonstrated by analysis of products obtained in incubations of linoleic acid with soybean and peanut lipoxygenases. Data of Table III in which the totals of linoleic acid hydroperoxides formed were corrected for nonenzymic oxidations in heat-inactivated enzyme incubations establish that the soybean lipoxygenase yields 13ZE-OOH as predominant enzymic product at pH 9.2 as expected, with but small amounts of the 13EE-OOH, 9EZ-OOH, and 9EE-OOH also formed. At lower pH enzyme regiospecificity is decreased somewhat although the 13ZE-OOH still predominates.

Peanut lipoxygenase oxidized linoleic acid to the 13ZE-OOH and 9EZ-OOH at pH 9.2, also with diminished regiospecificity at pH 5.5.

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