Preface

The first protocols book, *Free Radical and Antioxidant Protocols* ***(1)*** was published in late 1998. Sections were divided into three parts, covering selected biochemical techniques for measuring oxidative stress, antioxidant (AOX) activity, and combined applications. In choosing the 40 methods to be included in that book, I realized there were considerably more of equal value than that which we could have presented in a single volume. To produce a comprehensive resource, this book and a third are being compiled to expand coverage of the field.

A summary of papers ***(2)*** published on this important subject emphasizes the continuing rapid growth in oxidative stress investigations relating to our understanding of biochemical reactions, their relevance to pathophysiological mechanisms, how disease may arise, and how therapeutic intervention may be achieved ***(3)***. Although there is some overlap between the categories, the analysis shown below illustrates where current studies are concentrated and are almost evenly distributed between free radicals and AOX. Over the last 4 yr, there has been a 55% increase in the number of papers published in the area.

Table 1
Recent Citations of Oxidative Stress Biomarkers

	1997	**1998**	**1999**	**2000**
Free radical mechanisms	60	72	75	92
Free radicals in disease	78	88	109	111
AOX mechanisms	87	91	150	160
AOX in disease	94	122	155	204
Applications for treatment	0	2	5	8
TOTAL	**319**	**375**	**494**	**575**

Oxidative Stress Biomarkers and Antioxidant Protocols has added 33 more high-tech methods written by 73 authors from prestigious universities/institutes around the world, which together with our previous volume 108, provide a wide range of procedures for evaluating perturbations in cell function resulting from increased oxidative stress. Although primarily a reference for research, these two books also provide easy-to-follow directions that make them readily adapt-

able for academic use as a laboratory manual for graduate students in the basic sciences.

Of particular interest is the final chapter, which describes how the grouping of data from more than two biomarkers can be used to derive an appropriate statistical measure of change in the biological systems under study. The ability to more accurately interpret oxidative stress results in terms of either free radicals or AOX by using data from each to characterize laboratory or clinical observations, greatly enhances the value of this specific biostatistical approach.

I thank the Department of Small Animal Clinical Services, University of Florida College of Veterinary Medicine, and the Department of Clinical Laboratory Science, University at Buffalo for administrative support and facilities to produce this book. Professor John Walker, the *Methods in Molecular Biology*™ Series Editor, was helpful in the review process. Linda Rose and Chris Armstrong provided essential secretarial assistance and Aqeela Afzal compiled the literature search data shown in Table 1. I am indebted to authors in this volume and colleagues who alerted me to other technologies that were ultimately included to broaden its scope.

Donald Armstrong

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2

Simultaneous Determination of Polyunsaturated Fatty Acids and Corresponding Monohydroperoxy and Monohydroxy Peroxidation Products by HPLC

Richard W. Browne and Donald Armstrong

1. Introduction

Lipid peroxidation (LPO) is a prominent manifestation of free radical (FR) activity in biological systems. The primary target of FR attack on lipids is the 1,4-pentadiene structure of a polyunsaturated fatty acid (PUFA), which are either free or esterified to cholesterol or glycerol. Initiation occurs when a FR abstracts a methylene hydrogen from PUFA. In this reaction the FR is quenched and a PUFA centered alkoxyl radical (L•) is formed. L• then undergoes a spontaneous rearrangement of its double bonds forming a conjugated diene. Reaction of L• with molecular oxygen produces a PUFA-centered peroxyl radical (LOO•). Propagation occurs when either L• or LOO• act as initiating FR and attack a neighboring PUFA in a tightly packed lipid bilayer structure of a membrane or within a lipoprotein. The product of this reaction is a new L•, which can further propagate the reaction and form a lipid hydroperoxide (LHP) *(1)*. Termination occurs when an antioxidant (AOX) molecule capable of absorbing the intermediate free radicals, or free-radical scavengers, interrupts this chain reaction.

The hydroperoxide moiety of LHP can be reduced by divalent metal ions or glutathione-dependent peroxidases (phospholipid glutathione peroxidase or, following hydrolysis to free fatty acids, glutathione peroxidase) to an alcohol, yielding a hydroxy derivative (LOH). LHP and LOH represent the primary stable end products of lipid peroxidation *(2)*. Since biological samples are comprised many different LPO products can vary in carbon chain length and degree of unsaturation as well as regioisomerism of the position of the hydroperoxy

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or hydroxy group relative to the carbon chain ***(3)***. Furthermore, the native unoxidized PUFA composition of a system inherently effects the possible LPO products that are generated. Because of this, simultaneous determination of both the substrate and its derivative oxidation products has been suggested ***(4,5)***.

We have previously described a reverse-phase high-performance liquid chromatography (RP-HPLC) technique capable of separating regioisomeric species of LHP and LOH derived from the four major PUFA found in human plasma; linoleic, arachidonic, linolenic, and docosahexaenoic acid ***(6)***. Following total lipid extraction, alkaline hydrolysis and reextraction of the liberated fatty acids, two separate systems with different mobile-phase conditions and analytical columns were used, one for LOH and LHP and the second for the native unoxidized PUFA *(7)*. We report here on an alteration of this methodology allowing simultaneous determination of LHP, LOH, and PUFA on a single chromatographic separation.

This present methodology sacrifices a small amount of resolution of LHP and LOH for inclusive determination of PUFAs in a single isocratic run. Use of diode-array detection allows determination of the PUFA at 215 nm and the conjugated diene of LHP and LOH at 236nm. This method is useful for the determination of total LHP and LOH relative to their precursor PUFA within 20 min after injection.

2. Materials

2.1. Instruments and Equipment

2.1.1. Analytical HPLC System (Shimadzu Scientific Instruments, Columbia, MD)

1. Shimadzu LC-6A Pump.
2. Shimadzu SIL-7A Autosampler/injector.
3. Shimadzu SPD-M6A UV/VIS Photodiode Array.
4. PONY/IBM 486Dx PC.
5. Shimadzu CLASS-VP Chromatography Software.

2.1.2. HPLC Columns

1. Supelco LC-18 analytical, 4.6 × 250 mm, 5 micron particle size, 100 Å pore (Supelco, Bellafonte, NJ).
2. Supelcoguard C-18 (4.6 × 20 mm, 5 micron particle size, 100 Å pore).
3. Supelcoguard Guard Column Cartridge.

2.2. Reagents and Solvents

1. Unless otherwise indicated, reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

2. All organic solvents were HPLC grade and are obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Solvents were filtered through 0.22 micron, nylon, filter membranes immediately prior to use.

2.3. Standards

1. Linoleic (18:2ω6), linolenic (18:3ω3), arachidonic (20:4ω6), and docosahexaenoic acids (22:6ω6) and 5-hydroxy eicosatetraenoic acid methylester were purchased from Sigma in their highest purity.
2. Conjugated linoleic acid (CLA, iso-linoleic acid) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Calibration solutions were prepared by mass and dissolved in HPLC-grade ethanol and stored under argon at –80°C prior to HPLC analysis.
3. LHP standards were obtained as ethanolic solutions from Cayman (*see* **Note 1**).

3. Methods

3.1. LHP Standards

Calibration solutions are prepared in ethanol using a 160UV scanning spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) with the molar extinction coefficients provided by Caymen Chem. Co. **Table 1** lists the standards along with their shorthand nomenclature and molar extinction coefficients (*see* **Note 2**). Following individual standard peak identification, a hydroperoxy HPLC mixture is used on a daily basis to adjust for retention time fluctuations.

3.2. LOH Standards

LOH standards are prepared from LHP standards by methanolic sodium borohydried reduction as previously described ***(6)***. Following chloroform reextraction the LOH standards are dissolved in ethanol and calibration solutions prepared and stored as described for LHP. Following individual standard peak identification a hydroxy HPLC mixture is used on a daily basis to adjust for retention-time fluctuations (*see* **Note 3**).

3.3. HPLC Conditions

1. HPLC mobile phase consisted of 0.1% acetic acid/acetonitrile/tetrahydrofuran (41:41:18 v/v/v), which was premixed, filtered, and degassed under vacuum sonication. The mobile phase is continuously sparged with helium during analysis.
2. System flow rate is 1.3 mL/min and pressure of 175 Kg/cm^3.
3. The diode array monitored the column effluent from 200–300 nm with specific analysis channels of 236 and 215 nm with 8 nm bandwidth for the LHP/LOH and PUFA, respectively.

Table 1
Nomenclature, Retention Times and Molar Absorptivities of Polyunsaturated Fatty Acid Hydroperoxides and Hydroxy Derivative Standards

Retention time (min)	Name	Shorthand name	Absorbance maximum	Molar extinction coefficient
7.9	13-hydroxy-octadecatrieneoic acid	13-HOTE	235	23,000
8.9	13-hydroperoxy octadecatrieneoic acid	13HpOTE	235	23,000
9.8	13-hydroxy-octadecadieneoic acid	13-HODE	234	23,000
10.5	9- hydroxy-octadecadieneoic acid	9-HODE	234	23,000
11.3	13-hydroperoxy octadecadieneoic acid	13-HpODE	234	23,000
12.1	9-hydroperoxy octadecadieneoic acid	9-HpODE	234	23,000
12.2	15-hydroxy-eicosatetraenoic acid	15-HETE	236	27,000
13.1	12-hydroxy-eicosatetraenoic acid	12-HETE	237	27,000
13.5	12-hydroperoxy-eicosatetraenoic acid	12-HpETE	237	27,000
13.8	8-hydroxy-eicosatetraenoic acid	8-HETE	237	27,000
14.8	8-hydroperoxy-eicosatetraenoic acid	8-HpETE	237	27,000
15.3	5-hydroxy-eicosatetraenoic acid	5-HETE	236	27,000
16.4	5-hydroperoxy-eicosatetraenoic acid	5-HpETE	236	27,000
21.2	5-hydroxy-eicosatetraenoic acid methyl ester	Internal Standard (I.S.)	236	27,000
30.5	octadecatrieneoic acid (linoleic)	18:3	215	?
40.0	Docosahexaenoic acid	22:6	215	?
44.9	Eicosatetraenoic acid (arachidonic acid)	20:4	214	?
48.5	octadecadieneoic acid (linolenic acid)	18:2	209	?

3.4. Sample Extraction

1. EDTA plasma was collected into evacuated blood-collection tubes.
2. Hexane/isopropanol (HIP) total lipid extracts are prepared by adding 1.0 mL isopropanol to 0.5 mL ethylenediaminetetraacetic acid (EDTA) plasma.
3. Two mL of hexane is added, the vial perfused with nitrogen, capped, vortexed for 1 min, centrifuged for 3 min at 3,000*g* and the upper-hexane phase collected by aspiration (*see* **Note 4**). The extraction is repeated three times and the hexane layers are pooled and evaporated to dryness under nitrogen.
4. Alkaline hydrolysis of total dried lipid extracts are performed by dissolving in 0.95 mL of degassed, absolute ethanol. Fifty mL of 10 *M* sodium hydroxide (NaOH) is added, the sample perfused with nitrogen, capped, heated at 60°C for 20 min, and neutralized with 30 μL glacial acetic acid.
5. One hundred m_mL of 1.0 nmol/L 5-HETE-ME is added as internal standard. The ethanol is evaporated under nitrogen, the sample dissolved in 1.0 mL water, extracted twice with 2.0 mL of n-heptane, the upper phase collected and pooled, evaporated under nitrogen, and the residue dissolved in 250 m_mL of ethanol.

3.5. HPLC Analysis

1. Immediately prior to injection 250 μL of water is added to samples (*see* **Note 5**). One hundred fifty m_mL of the sample is injected into the HPLC system, eluted isocratically with mobile-phase conditions described in **Subheading 3.3., step 1**, over 60 min and monitored at 200–300 nm by the photodiode array (*see* **Note 6**).
2. Quantification is based on an external calibration curve using ethanolic standards prepared on a Shimadzu 160 UV scanning spectrophotometer applying the max and absorptivity coefficients provided by the manufacturer. Serial dilutions are made in ethanol/water 50:50 (v/v) and standard curves generated by triplicate injections of each calibrator. Sample concentrations are interpolated from standard curves and corrected for recovery of the 5-HETE-ME internal standard (*see* **Note 3**).

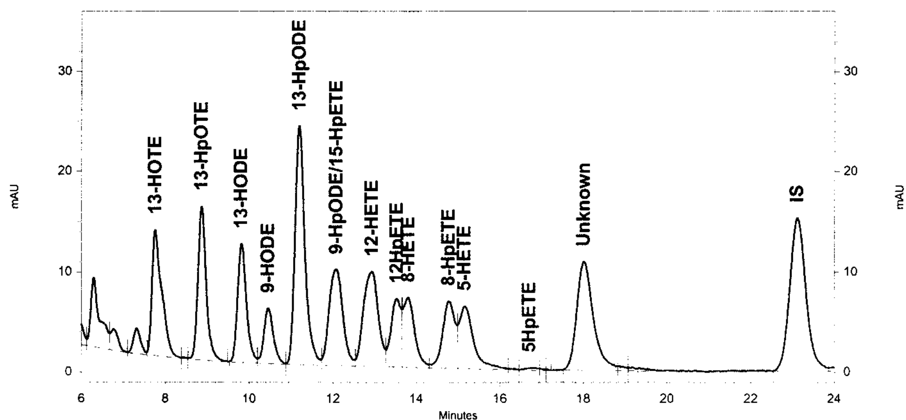
3.6. Results

Figure 1 shows two displays of the chromatographic data from a mixed preparation of standards. **Figure 1A** shows the two dimensional graph of a slice through the diode array three dimentional data at 236 nm identifying LHP and LOH peaks. **Figure 1B** shows the slice at 215 nm identifying the native PUFA. **Figure 2** shows chromatographic data obtained from a human plasma sample prepared as described in **Subheading 3.4.**

4. Notes

1. LHP and LOH standards can be purchased or synthesized. The synthesis of standards is described in detail in volume 108 of this series *(8)*.
2. If LOH standards are synthesized by methanolic sodium borohydried reduction, rather than purchased in purified form, it is necessary to perform calibrations of

A



B

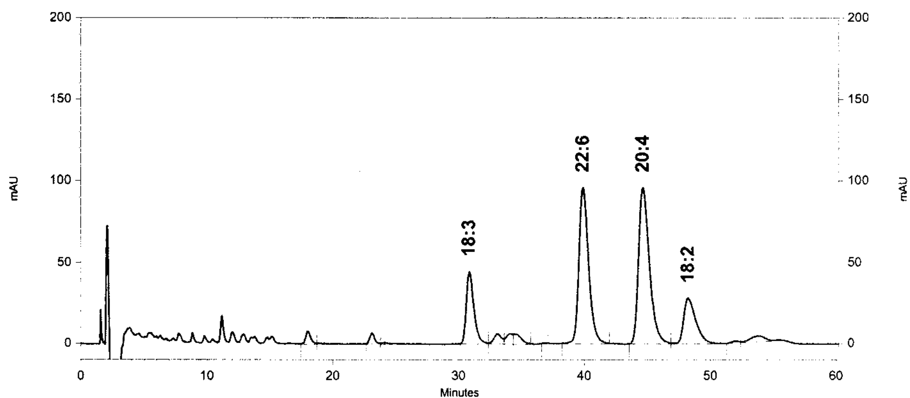


Fig. 1. Simultaneous chromatograms of LHP and LOH standards at 236 nm **(A)** and native unoxidized PUFA standards at 25 nm **(B)**. *See* **Subheading 3.3.** for HPLC conditions and **Table 1** for nomenclature of peaks.

LHP and LOH separately since trace amounts of the sodium borohydried in the LOH preparation may reduce LHP upon mixing.

3. It is critical that all solvents, especially those used for extraction, are thoroughly degassed to remove dissolved oxygen and prevent lipid oxidation during processing. We routinely accomplish this by placing solvents in an ultrasonic water bath and applying a vacuum followed by 15 min of helium sparging. Screw-cap extraction vials are perfused with nitrogen or argon and immediately capped prior to vortexing or incubations.

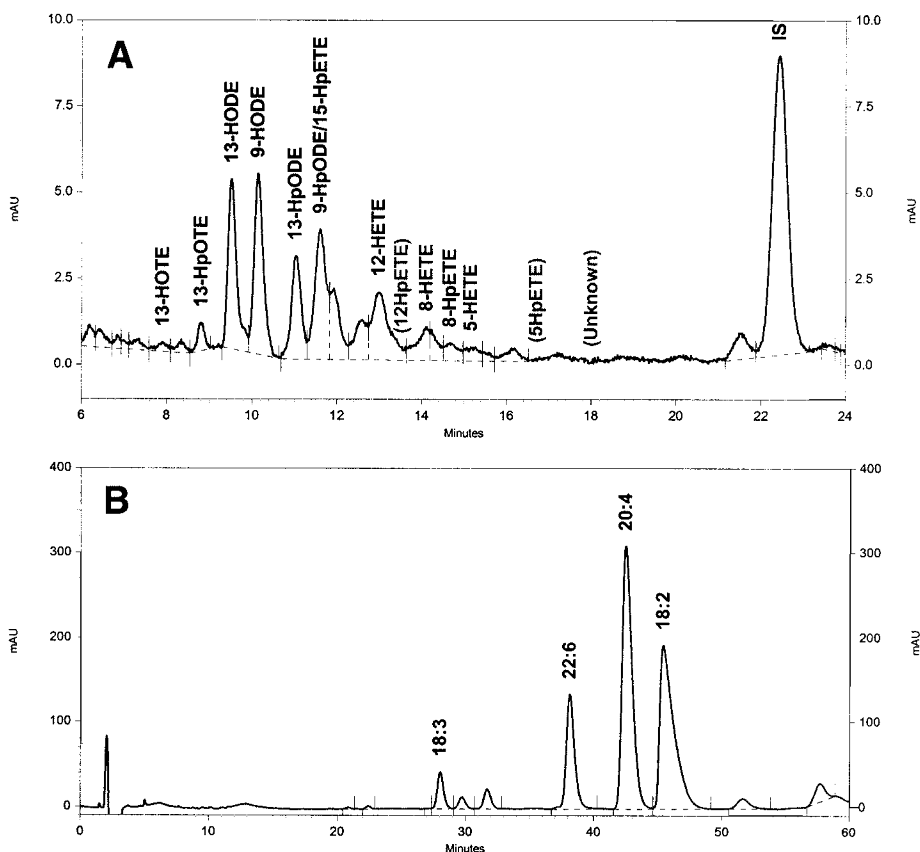


Fig. 2. Simultaneous chromatograms of LHP and LOH at 236 nm **(A)** and native unoxidized PUFA at 215 nm **(B)** isolated from human plasma by total lipid extraction, and saponification.

4. Samples need to be injected in a solution that is at least 50% water in order to ensure good mass transfer of the sample to the stationary phase. Samples injected in pure solvent such as ethanol give extremely broad peaks and poor resolution.
5. It should be noted that a photodiode array is not necessary for this methodology and a simple two-channel UV detector could be used. Integration of peak areas is performed at 236 nm with an 8 nm bandwidth for LOH and LHP. This wavelength and bandwidth are chosen to encompass the wavelength of maximum absorbance (I_{max}) of the ODEs at 234 nm and ETE at 237 nm.

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