ELSEVIER

Journal of Chromatography B, 671 (1995) 223–236

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Analytical technologies for lipid oxidation products analysis

Michael Kinter

Departments of Microbiology and Pathology, University of Virginia, Health Sciences Center, Box 441, Charlottesville, VA 22908, USA

Abstract

Productive investigation of the contribution of oxidative stress to human disease is facilitated by the design and application of suitable analytical technologies for oxidation product analysis. Lipid oxidation, including polyunsaturated fatty acid and cholesterol oxidation, produces a variety of products that can function as indexes of the extent of oxidation. These products include fatty acid hydroperoxides and hydroxides, aldehydes, prostanoids, hydrocarbons, and cholesterol hydroperoxides and hydroxides, epoxides, and carbonyls. Some of these oxidation products have biological activities that can contribute to tissue damage in unique ways. This paper reviews the state-of-the-art for chromatographic analysis of these products through a discussion of advances that have taken place since 1990.

Contents

1. Introduction

Damage to cells and tissues by free radical species is hypothesized to contribute to numerous pathological conditions. A series of recent reports have reinvigorated these studies. Work by Steinberg et al. [1] showing that the oxidation of low density lipoprotein (LDL) produces structural changes which accelerate lipid deposition in the arterial wall, work by Deng et al. [2] showing that a mutation in the human Cu,Zn superoxide dismutase gene in patients with familial amyotrophic lateral sclerosis produces a form of the enzyme with reduced activity, and work by Stadtman [3] showing that aging cells contain oxidatively modified forms of certain enzymes

0378-4347/95/$29.00
SSDI 0378-4347(95)00189-1

has detailed clear links between oxidative stress and important human pathologies.

This reinvigoration of interest in the contribution of oxidative stress to tissue damage has also produced new demands on analytical technologies used for both qualitative and quantitative analyses of oxidative damage to biological systems. Oxidative damage to lipids, generally referred to as lipid peroxidation, is one type of damage. The purpose of this paper is to review the state-of-the-art for the analysis of the products of free radical mediated oxidation of both polyunsaturated fatty acids and cholesterol through a discussion of advances that have taken place since 1990. The review is limited to chromatographic methods applicable to the study of biological systems.

The reader should be aware of the inherent difficulty faced in designing a measurement system to assess oxidative damage to lipids in biological systems. Analysts are generally familiar with the process of evaluating analytical protocols in terms of performance factors such as accuracy, precision, sensitivity, limits of detection and potential sources of interferences. Often overlooked, however, is the importance of selecting analytes with an understanding of both reactions that lead to a given compound's formation *and* reactions that contribute to subsequent metabolism. Identification of new metabolites of the primary products of lipid oxidation and development of a suitable quantitative method are important areas for future research especially for applications in increasingly complex in vitro systems and in vivo.

2. Polyunsaturated fatty acid oxidation products

Fatty acid oxidation, specifically polyunsaturated fatty acid oxidation, is the most extensively studied component of oxidative damage to biological systems. Saturated, monounsaturated and polyunsaturated fatty acids are all integral components of cellular membranes. These different classes of fatty acids possess a variety of physical and chemical properties that are incorporated into membranes to achieve the properties of that membrane. The double bond systems in polyunsaturated fatty acids, however, contain *bis*-allylic hydrogens with bond energies that make them susceptible to hydrogen abstraction reactions that initiate and propagate a chain reaction oxidation scheme known as autoxidation. As seen in Fig. 1, autoxidation produced an intriguing array of compounds many of which can also be produced by enzymatic activities, such as cyclooxygenase and lipoxygenase activities, that have been associated with normal cellular physiology. The products of polyunsaturated fatty acid oxidation addressed in this review are listed in Table 1. This list illustrates the complexity of polyunsaturated fatty acid oxidation chemistry and the disparate chemistry of the respective products. Although investigations of fatty acid oxidation frequently rely on analysis of a single product, it is clearly beneficial to monitor as many products as is feasible. An additional consideration in the design of methods suitable for studying fatty acid oxidation is the interdependence of the reactions which lead to the individual products and the fact that quantitation

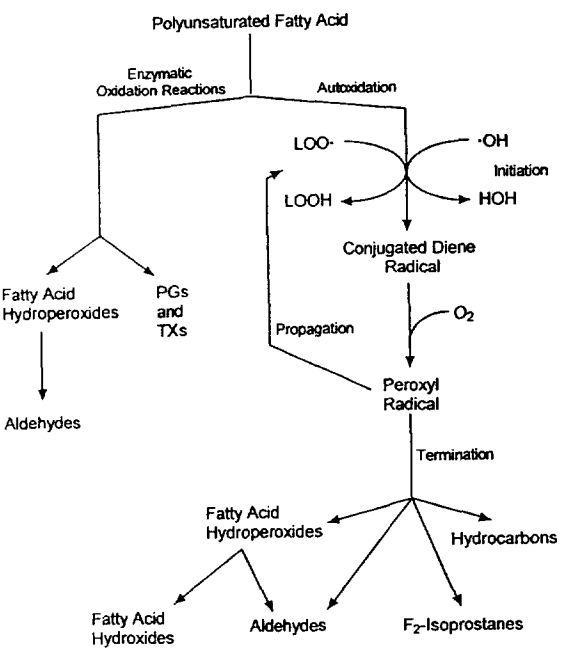

Fig. 1. Summary of the reaction chemistry associated with polyunsaturated fatty acid oxidation.

Table 1
Summary of the different types of polyunsaturated fatty acid oxidation products

Fatty acid hydroperoxides A series of regioisomers, free and esterified into triglycerides, phospholipids and cholesterol esters
Fatty acid hydroxides A series of regioisomers, free and esterified into triglycerides, phospholipids and cholesterol esters
Aldehydes For example malondialdehyde, acetaldehyde, formaldehyde, 4-hydroxy-2-nonenal, hexanal
Non-cyclooxygenase prostanoids A series of regioisomeric F_2-isoprostanes, free and esterified into phospholipids
Hydrocarbons Ethane, pentane

of one product may or may not accurately reflect the formation of any other product.

2.1. Fatty acid hydroperoxide analysis

The immediate products of polyunsaturated fatty acid oxidation are fatty acid hydroperoxides. It is reasonable to believe, based on this immediacy, that fatty acid hydroperoxides should be the most abundant class of fatty acid oxidation products although quantitative comparisons among the classes of products have not been made. A characteristic of the formation of hydroperoxides from polyunsaturated fatty acids is the generation of a conjugated diene as the double bonds rearrange. This rearrangement provides one approach to monitoring the formation of polyunsaturated fatty acid hydroperoxides, the measurement of absorbance at 234 nm versus an appropriate blank [4]. Other methods for fatty acid hydroperoxide quantitation such as iodometric titration [5], xylenol orange reactivity [6] and glutathione oxidation [7] have also been used. While these approaches have worked well in many simpler systems, they lack sensitivity and specificity for application in complex systems and provide no information about specific hydroperoxides. These deficiencies have lead to the development of a variety of chromatographic approaches for determining polyunsaturated fatty acid hydroperoxides in biological systems utilizing both liquid and gas chromatography.

One series of methods for fatty acid hydroperoxides are based on liquid chromatography. The use of HPLC avoids the challenges of preparing the relatively labile hydroperoxides for gas chromatography. As can be seen in a number of these methods, HPLC methods also allow the direct analysis of esterified forms of the hydroperoxides. An effective approach to increasing both the sensitivity and specificity of hydroperoxide analysis has been to use post-column chemiluminescence reactions. Chemiluminescence systems employ luminol or isoluminol with either microperoxidase [8–10] or cytochrome *c* [11–13] as the source of the heme iron metal catalyst. The analytes are isolated by solvent extraction of the total lipids. Both reversed-phase chromatography with methanol–water based mobile phases and silica chromatography with chloroform–methanol based mobile phases have been used with good result to quantitate the hydroperoxides in the different phospholipid classes and cholesterol esters. One analysis of fatty acid hydroperoxides esterified in either phosholipids or cholesterol esters using chemiluminescence detection is illustrated in Fig. 2. These methods have been applied to plasma [9], tissues [12,14] and lipid emulsions [15] with limits of detection as low as 0.1 pmol on column.

Post-column reactions combined with fluorimetric detection have also been used to enhance the detectability of hydroperoxides. Diphenyl-1-pyrenylphosphine reacts with the hydroperoxides to form the corresponding oxide that is detected by fluorescence at 380 nm with excitation at 352 nm [16,17]. For this method, the lipids were extracted into hexane and analyzed using a silica column eluted with a linear gradient of 1-butanol in hexane to separate hydroperoxides esterified in triglycerides and cholesterol esters in plasma at nmol/l concentrations [17]. These investigators subsequently modified this method to allow simultaneous determination of phosphatidylcholine, triglyceride and choles-

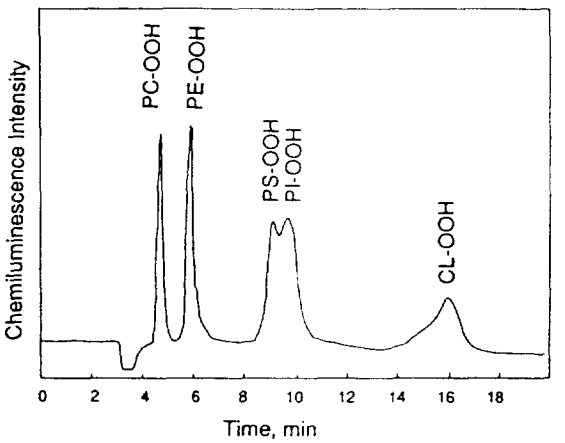

Fig. 2. HPLC analysis of 10 pmol of phospholipid hydroperoxides with chemiluminescence detection. Standard fatty acid hydroperoxides esterified in phosphatidyl choline (PC-OOH), phosphatidyl ethanolamine (PE-OOH), phosphatidyl serine (PS-OOH), phosphatidyl inositol (PI-OOH), and cholesterol ester (CH-OOH) are separated using a 25 cm × 4.6 mm I.D., 5-μm Supelco aminopropyl column eluted with a methanol–*tert.*-butanol–40 m*M* monobasic sodium phosphate mobile phase with detection by isoluminol chemiluminescence. (From Ref. [10], used with permission.)

terol ester hydroperoxides by switching the eluent from a short silica column onto either a reversed-phase column, to chromatograph the phosphatidylcholine hydroperoxides, or a second silica column, to chromatograph the triglycerides and cholesterol ester hydroperoxides [18]. These hydroperoxides were quantitated in human plasma at nmol/l levels using this method.

Other investigators have used HPLC methods with detection by absorbance at 234 nm. The reduction products of the hydroperoxides formed by 15-lipoxygenase activity, 13-hydroxy-octadecadienoic and 15-hydroxyeicosatrienoic acid, have been quantitated by reversed-phase HPLC eluted with a tetrahydrofuran–acetonitrile–water–acetic acid (22:40:38:0.05, v/v) mobile phase [19]. Both the free and esterified forms of these hydroxy fatty acids were determined in vascular tissues by making the measurements with and without saponification of the fatty acid esters. The analysis of fatty acid hydroperoxides formed in the oxidation of low-density lipoprotein by HPLC using a silica column eluted with a heptane–diethyl ether–isopropanol–acetic acid (100:15:0.35:0.1, v/v) mobile phase is shown in Fig. 3 [20]. In this experiment, the esterified products were saponified to yield free fatty acid hydroperoxides that were recovered by solvent extraction for analysis.

Other methods for determining of fatty acid hydroperoxides utilize gas chromatography with mass spectrometric detection. The most important advantage of GC–MS methods is enhanced sensitivity. The disadvantage of these methods is the complex derivatization protocols required to make the analytes amenable to GC–MS analysis. In the case of fatty acid hydroperoxides, the functional groups which require attention are the hydroperoxide/hydroxyl group, the carboxylic acid, and the double bond system. A typical derivatization protocol for fatty acid hydroperoxides involves reduction of the double bonds and hydroperoxides, saponification of the glycerol and cholesterol esters, esterification of the carboxylic acid, and silylation of the hydroxyl group [21–23]. A recent report describes the direct silylation of hydroperoxides [24], thereby simplifying this series of steps to some degree. These analyses have used a DB-5 column programmed from 260°C to 310°C [22] or a DB-1 column programmed from 50°C to 285°C [23] with GC–MS detection. Despite the complexity of the derivatization and analysis protocols, the increase in sensitivity obtained using GC–MS analyses is substantial and allows the analyses to be performed on specimens that cannot be analyzed by other methods.

2.2. Aldehyde analysis

A number of aldehydes have been identified associated with oxidative stress. These aldehydes are generally believed to result from the breakdown of polyunsaturated fatty acid hydroperoxides, although detailed chemical mechanisms have not been well described. As can be seen in the discussion below, analytical method development has focused on malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4HNE), with

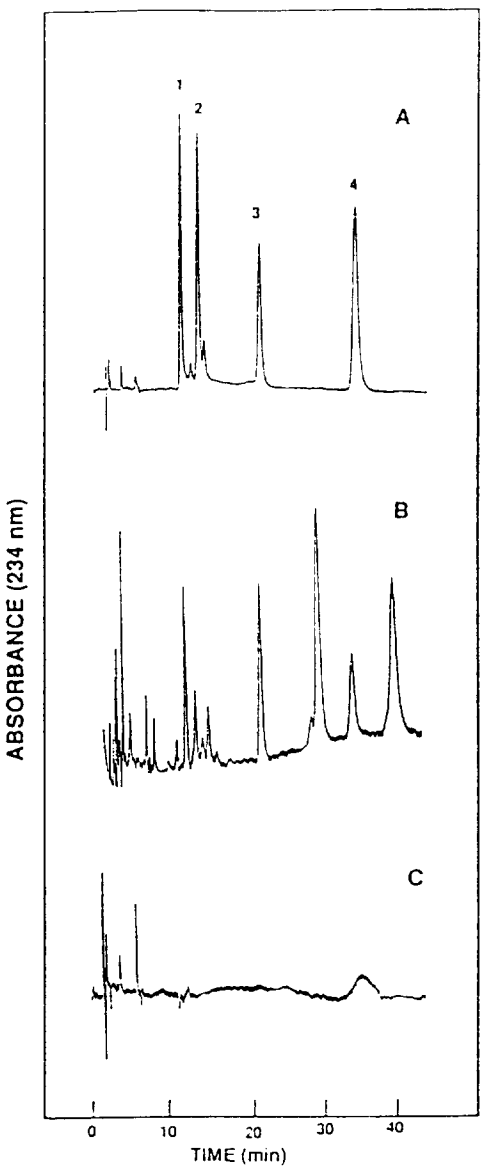

Fig. 3. HPLC analysis of conjugated dienes extracted from low-density lipoprotein (LDL). (A) Analysis of a standard containing, 13-hydroperoxyoctadecadienoic acid (peak 1), 9-hydroperoxyoctadecadienoic acid (peak 2), 13-hydroxyoctadecadienoic acid (peak 3), and 9-hydroxyoctadecadienoic acid (peak 4). (B) Analysis of LDL oxidized by a 4-h exposure to 5 μM $CuSO_4$. (C) Analysis of unoxidized LDL. Each analysis was performed using a 25 cm × 4.6 mm I.D., 5 μm, Zorbax Sil column eluted with a heptane–diethyl ether–isopropanol–acetic acid (100:15:0.35:0.1, v/v) mobile phase at 1.75 ml/min with detection by absorbance at 234 nm. (From Ref. [20], used with permission.)

additional work detecting and quantifying hexanal, formaldehyde and acetaldehyde.

2.2.1. Malondialdehyde

The quantitation of MDA has contributed greatly to investigations of the basic chemistry and biochemistry of polyunsaturated fatty acid oxidation. These studies initially utilized the reaction of MDA with thiobarbituric acid (TBA) to form an adduct with an absorbance maximum at approximately 535 nm and molar extinction coefficient in the range of 155 000 M^{-1} cm^{-1}. The formation of this product is used in a spectroscopic assay for MDA that has been applied in innumerable investigations. Closer scrutiny of this test, however, began to show that a number of compounds other than MDA also react with TBA to produce adducts which adsorb at approximately 535 nm. As a result, chromatographic analyses were developed for the MDA–TBA adduct so that specific detection could be assured.

Chromatographic quantitation of the MDA–TBA adduct has been applied to specimens of lung lavage fluid [25], plasma [26–28], cultured cells [27] and urine [29]. For these assays, MDA–TBA adducts are formed by treating the samples with TBA in an acidic solution at 100°C for 1 h. The assays utilize reversed-phase chromatography with methanol–phosphate buffer mobile phases and detection of the TBA adducts accomplished by monitoring absorbance at 532 nm [25,27–29] or fluorescence with excitation at 532 nm and emission at 553 nm [26,30]. The detection of MDA in plasma as the TBA adduct is shown in Fig. 4.

A second problem associated with MDA methods based on TBA adduct formation is that MDA may be formed by the breakdown of lipid hydroperoxides under the conditions used for adduct formation. As a result, the levels of MDA that are measured in these assays are sometimes referred to as an index of lipid peroxide levels. However, this lack of specificity has lead investigators to develop quantitative methods for MDA which utilize detection schemes that avoid these harsh reaction conditions. One approach

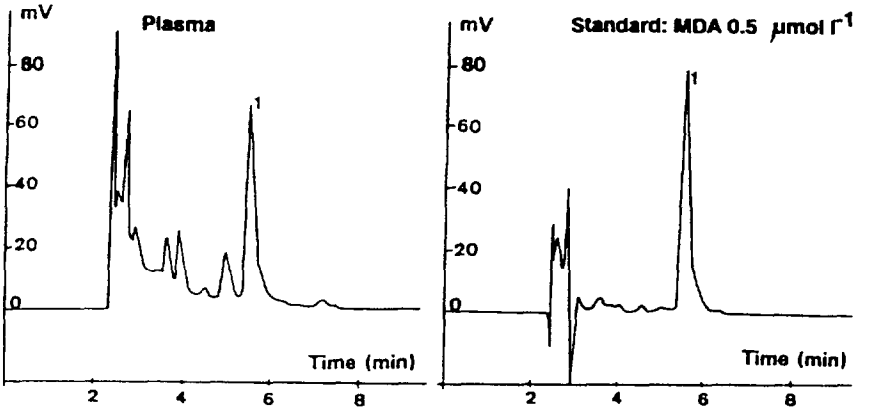

Fig. 4. HPLC analysis of malondialdehyde–thiobarbituric acid adduct (peak 1) in plasma. The analysis was carried out using a 25 cm × 4.6 mm I.D., 5 μm, Beckman ODS column eluted with a methanol–50 m*M* phosphate buffer, pH 6.0 (42:58, v/v) mobile phase at 1.0 ml/min with detection by absorbance at 532 nm. (From Ref. [27], used with permission.).

being used is ion-pair chromatography with tetradecyltrimethylammonium bromide as the ion-pairing reagent [31–33]. Sample preparation for this method involves steps associated with protein precipitation and solid-phase extraction but does not utilize any high-temperature steps. The free MDA is determined by reversed-phase HPLC using a solvent system that contains the ion-pairing reagent with detection achieved by monitoring absorbance at 267 nm. Free MDA has also been quantified by size-exclusion chromatography using a Spherogel-TSK G1000 PW column eluted with a 0.1 *M* phosphate buffer, pH 8 mobile phase and detection by monitoring absorbance at 267 nm [34–36]. As with the ion-pairing chromatography method, sample preparation for the size-exclusion method does not utilize any high-temperature steps which might convert lipid hydroperoxides to MDA. Other methods for MDA determinations utilize the formation of the dinitrophenylhydrazone derivative for reversed-phase HPLC analysis [29,37–40]. The derivative is formed by treating the samples with 2,4-dinitrophenylhydrazine at room temperature, and isolating the products by extraction with pentane [37,38] or chloroform [29]. The extracts are analyzed using reversed-phase HPLC using acetonitrile–0.1 *M* HCl (45:55, v/v) [29], acetonitrile–water–acetic acid (40:60:0.1, v/v/v) [37], or acetonitrile–water (49:51, v/v) [38] mobile phases with the hydrazones detected by monitoring absorbance in the 300 nm to 330 nm range. As can be seen the subsequent section, one advantage of this approach that it can allow for simultaneous analysis of other aldehydic products of polyunsaturated fatty acid oxidation.

Gas chromatographic methods for the quantitation of MDA have also been reported. One approach has been to reduce MDA to 1,3-propane diol with borane trimethylamine [41]. The diol product is then converted to its *tert.*-butyldimethyl silyl ether and analyzed by GC–MS with a 25-m HP5 capillary column programmed from 115°C to 165°C and 1,3-[$^2H_8$]propanediol used as the internal standard. A detection limit of 0.5 nmol/sample was reported. MDA can also be converted to 1-methylpyrazole by reaction with N-methylhydrazine at room temperature for 1 h [42,43]. This derivative is recovered by extraction with dichloromethane and analyzed by gas chromatography with a 30-m DB-WAX capillary column programmed from 30°C to 200°C and a nitrogen–phosphorus detector. Quantitation uses N-methylacetamide as the internal standard. One advantage of the GC methods is increased sensitivity, particularly when used with mass spectrometric detection. The second advantage of the GC methods is that simultaneous analysis of several aldehydes can be achieved.

2.2.2. Other aldehydes including 4-hydroxy-2-nonenal

Other aldehydes have been identified as products of polyunsaturated fatty acid oxidation. Interest in these other aldehydes as possible indexes of polyunsaturated fatty acid oxidation has increased as an appreciation of the limitations in the quantitative methods for MDA has grown. However, selected aldehydes can also possess biological activities that merit investigations focused on issues beyond simple monitoring of polyunsaturated fatty acid oxidation. As an example, 4HNE has been hypothesized to contribute to many of the deleterious processes associated with the damaging effects of oxidative stress. 4HNE has been found to be cytotoxic and cytostatic, to be capable of inhibiting enzymes via covalent modification and to be capable of covalently modifying LDL in a manner which increases its atherogenicity. As a result, quantitation of compounds such as 4HNE may be useful as both a general index of the extent of polyunsaturated fatty acid oxidation and a direct measure of biologically active xenobiotics that substantially contribute to tissue damage.

Methods for the simultaneous determination of formaldehyde, acetaldehyde and malondialdehyde based on the formation of dinitrophenylhydrazone derivatives have been described by several groups [37–40,44–47]. The dinitrophenylhydrazone derivative is formed by treating the samples with dinitrophenylhydrazine at room temperature, and isolating the products by extraction. As shown in Fig. 5, the extracts are analyzed using reversed-phase chromatography with acetonitrile–water–acetic acid (40:60:0.1, v/v/v) as the mobile phase and the hydrazones detected by monitoring absorbance in the 300 nm to 330 nm range. The assays have been applied to heart perfusates [37,46] and urine [38–40,44,45,47] to detect these aldehydes at levels ranging from 1 to 6 nmol/ml. It important to note that experiments utilizing these methods have successfully documented increases in these analytes in the urine of rats treated with carbon tetrachloride [40] and paraquat [38], two compounds that have been shown to enhance lipid peroxidation in in vitro experiments.

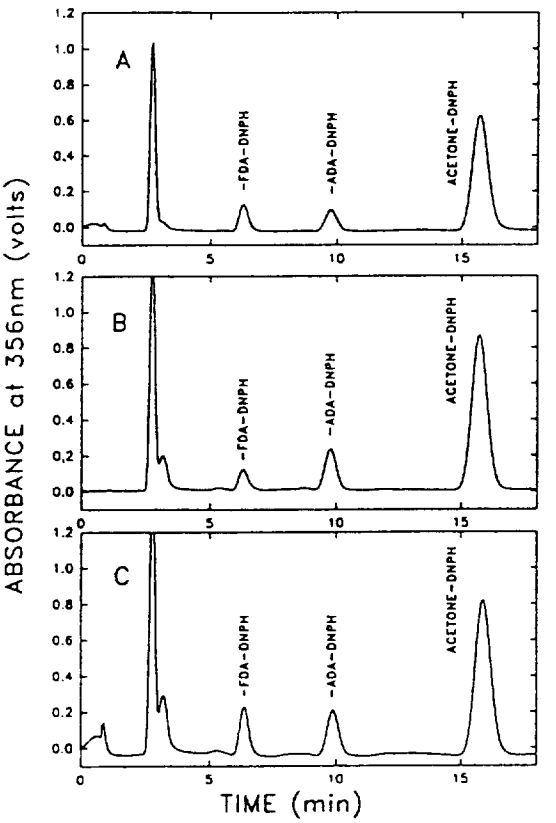

Fig. 5. HPLC analysis of dinitrophenylhydrazone derivatives of formaldehyde (FDA), acetaldehyde (ACA), and acetone in heart perfusates. (A) Analysis of the baseline perfusate. (B) Analysis of the perfusate obtained after 30 min of ischemia and 15 min of reperfusion. (C) Analysis of the perfusate obtained after 30 min of ischemia and 60 min of reperfusion. All analyses were carried out using a 7.5 cm × 4.6 mm I.D., 3-μm Beckman Ultrasphere ODS column eluted with an acetonitrile–water–acetic acid (40:60:0.1, v/v/v) mobile phase at 1 ml/min with detection by absorbance at 356 nm. (From Ref. [37], used with permission.)

The dinitrophenylhydrazone derivatization of 4HNE has also been used for analysis by HPLC [48–50] and TLC [51]. These methods also form the DNPH derivative at room temperature and isolate the product by extraction. Preparative TLC using silica gel eluted with dichloromethane has been used by some investigators to fractionate the aldehydes into 4-hydroxy-2-alkenals and non-hydroxylated aldehydes including saturated aldehydes such as hexanal and unsaturated aldehydes such as 2,4-nonadienal [48,50]. The two

fractions are analyzed separately using reversed-phase HPLC with a methanol–water (4:1, v/v) mobile phase and detection by absorbance at 350 nm. Other investigators have used electrochemical detection with reversed-phase HPLC for the determination of 4HNE as its DNPH derivative [49]. These authors find that electrochemical detection provides additional sensitivity for this analysis. The 4HNE–DNPH derivative has also been detected by densitometric determinations of absorbance at 360 nm on silica gel thin-layer chromatography plates eluted with dichloromethane [51]. This method quantitates the aldehydes collectively as either alkanal or hydroxyalkenal species at levels in the nmol/sample range. These investigators also describe the use of cyclohexadione derivatives of aldehydes for reversed-phase TLC eluted with methanol–water (70:30, v:v) and densitometric analysis with fluorescence detection.

Analytical methods for 4HNE based on gas chromatography have also been utilized for the analysis of biological samples. The primary advantage of GC assays are the increased sensitivity that is obtained particularly when mass spectrometric detection is used. The primary disadvantage is the increased sample processing and derivatization required for GC analysis. One approach taken that did not use mass spectrometric detection formed methylhydrazine derivatives of the aldehydes for detection with a nitrogen–phosphorus detector [42,43]. In this method, 2-methylpyrazine was added as an internal standard and the samples analyzed with a 30-m DB-WAX column programmed from 30°C to 200°C. Concentrations of nine different aldehydes including 4HNE were detected in oxidized oils with the 4HNE levels ranging from 0.05 to 0.1 μg/mg of arachidonic acid. Other investigators report the reduction of 4HNE and hexanal to the corresponding alcohols with $NaBH_4$ which are then silylated to form the *tert.*-butyldimethylsilyl ethers [41]. 4-Hydroxy-2,3-[$^2H_2$]-2-nonenal is added to the samples as an internal standard and the samples analyzed using a 25-m HP-5 capillary column monitoring the respective $[M-57]^+$ ions by mass spectrometry. The limit of detection was found to be 0.5 nmol/sample. Gas chromatographic resolution of the two 4HNE enantiomers has been accomplished using a permethyl-β-cyclodextrin capillary column [52]. The aldehyde moiety is not derivatized for this analysis but the hydroxy group is acetylated using acetic anhydride; other derivatization reactions were tested but did not allow the enantiomeric resolution.

It is clear that 4HNE is an active xenobiotic with potential processing through a number of metabolic routes. The methods described above are all designed to detect 4HNE in its free form. One disposition of 4HNE is conjugation to glutathione with subsequent conversion to mercapturic acids in appropriate situations. 4HNE–mercapturic acid conjugate has been determined using GC–electron capture negative ion chemical ionization MS [53]. This protocol isolates the mercapturic acids from urine by extraction and converts them to the pentafluorobenzyl esters for analysis. Other dispositions of 4HNE are covalent binding to proteins by 1,2-addition reactions (also referred to as Schiff base formation) with lysine residues, 1,4-addition reactions with lysine and histidine residues, and through thioether linkages to cysteine residues. 4HNE can be released from the Schiff base linkages by reaction with hydroxylamine [54]. GC–MS methods have been described using derivatization of the aldehyde moiety with hydroxylamine with subsequent conversion to the bis-*tert.*-butyldimethylsilyl derivative [54,55]. Cyclohexanone [55] and hydroxynonanal [54] were used for internal standards and the samples analyzed by capillary gas chromatography with mass spectrometric detection monitoring the $[M-57]^+$ ions. Limits of detection as low as 5 pmol/sample have been reported using these methods [54]. An LC–MS method using thermospray ionization has been used for the pentafluorobenzyl oxime derivative of 4HNE [56]. Tissue homogenates were analyzed using reversed-phase HPLC with mass spectrometric detection of negative ions formed by thermospray ionization and 4HNE detected at levels less than 1 nmol/sample. Covalent 4HNE–protein adducts have also be detected following acid hydrolysis of proteins [57]. For this analysis, the proteins are first

treated with $NaBH_4$ to stabilize the adducts and hydrolyzed with hydrochloric acid. The amino acid mixture is analyzed by reversed-phase HPLC using fluorescence detection with excitation at 340 nm and emission at 450 nm. A pre-column derivatization scheme is used to form the *o*-phthalaldehyde derivatives and enhance detectability. The challenge of the chromatography is to separate the modified histidine and lysine moieties from far greater quantities of leucine and lysine.

2.3. F_2-Isoprostane analysis

A series of endoperoxide species formed by the cyclization of fatty acid hydroperoxides were first described by Porter and Funk [58]. Subsequent work by Morrow and co-workers [59–62] have begun to characterize the formation of several isomeric reduction products of these endoperoxides containing a ring group characteristic of F-type prostaglandins that they refer to as F_2-isoprostanes. These investigators have shown that F_2-isoprostanes are produced by free radical oxidation of arachidonic acid and arachidonic acid containing phospholipids [60,63] and that they are formed in humans by non-cyclooxygenase mediated processes [59]. As a result, the potential utility of the F_2-isoprostanes for monitoring oxidative stress in vivo is most exciting. Two important possible limits to this utility, pointed out by these investigators [61], that should be considered are the potential for adventitious production of the analytes in sample handling and processing steps, and the fact that relatively high basal levels may make increased or decreased production difficult to detect.

The analysis protocols used for the detection and quantification of the F_2-isoprostanes are based on the method described by Morrow et al. [60] that uses gas chromatography with 15-m OV-17 capillary column and negative ion chemical ionization mass spectrometric detection. The analysis of F_2-isoprostanes in human plasma by this method is shown in Fig. 6. Similar techniques have been used for other prostaglandins, thromboxanes and leukotrienes, primarily to take advantage of the high sensitivity of negative ion chemical ionization mass spectrometry (NICIMS). For the analysis of the F_2-isoprostanes, the selectivity of analysis obtained by the combination of pentafluorobenzyl ester formation and NICIMS detection would appear to be the more important benefit of this analytical approach.

These assays utilize stable isotope labeled internal standards such as [$^2H_7$]-9α,11β-prostaglandin F_2 [61], [$^2H_4$]prostaglandin $F_{2\alpha}$ [63,64] and [$^{18}O_4$]-9α,11β-dihydroxy-15-oxo-2,3,18,19-tetranorprost-5-ene-1,20-dioic acid [62]. The use of stable-isotope dilution in these protocols is ideal because the samples must undergo extensive fractionation to isolate the F_2-isoprostanes. In all the assays, the lipid species are recovered by a total lipid extraction. Free and esterified species can be determined by the difference between quantities detected in samples extracted directly and samples extracted after the phospholipid species are hydrolyzed by either base-catalyzed saponification [63–67] or enzymatic hydrolysis with phospholipase A_2 [65]. The lipids are fractionated by two-step combinations of ODS column solid-phase extraction (SPE) followed by thin-layer chromatography or ODS column SPE followed by NH_2 column SPE to yield the purified analytes. The F_2-isoprostanes are converted to the pentafluorobenzyl ester, trimethylsilyl ether derivatives and gas chromatographic analysis carried out using either a 15- or 30-m DB1701 or OV-17 capillary column with temperature programming and detection by negative ion chemical ionization. Blood [59,63,66], urine [59], LDL solutions [63,64], and liver tissue [65–67] have all been studied. The levels of F_2-isoprostanes detected in these studies, frequently expressed as a total of all regioisomers, range from 5 to 30 pg/ml in normal plasma, 500 to 4000 pg/mg creatinine in urine, 0.1 to 10 ng/mg protein in LDL depending on the extent of oxidation, and 4 to 10 ng/g tissue in liver tissues.

2.4. Hydrocarbon analysis

Two hydrocarbons have been identified as products of polyunsaturated fatty acid oxidation;

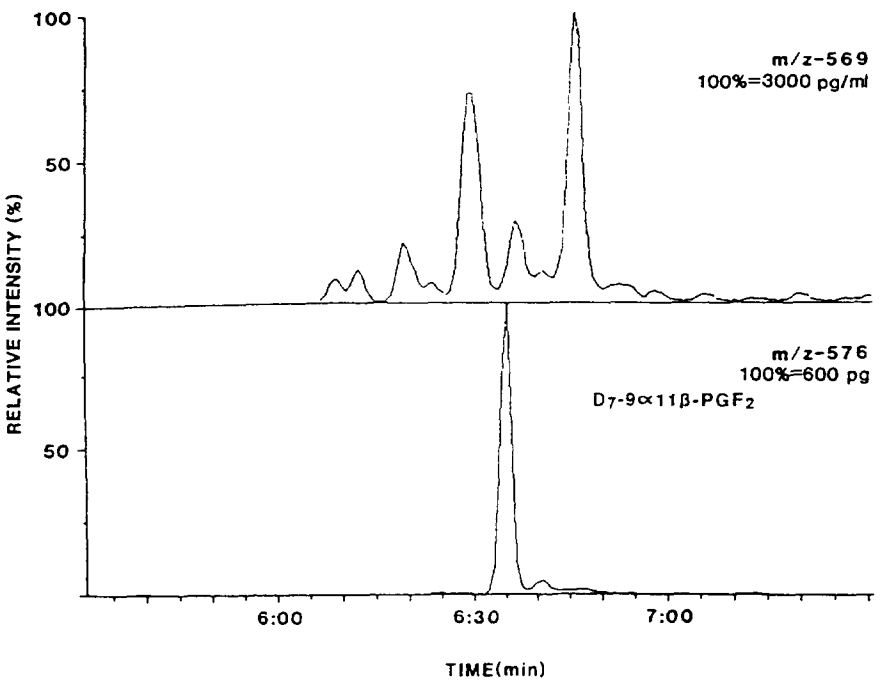

Fig. 6. GC–MS analysis of F_2-isoprostanes in human plasma. The analysis was performed following treatment to form the pentafluorobenzyl ester, trimethylsilyl ether derivatives by capillary gas chromatography with negative ion chemical ionization mass spectrometric detection. The F_2-isoprostanes are detected in the m/z 569 ion chromatogram while the [$^2H_7$]-9α,11β-prostaglandin F2 internal standard is detected in the m/z 576 ion chromatogram. (From Ref. [60], used with permission.).

ethane from $n-3$ polyunsaturated fatty acids, and pentane from $n-6$ fatty acids. The analysis of ethane and pentane in biological systems has the primary advantage of being non-invasive which is particularly advantageous in studies of animals and humans. The primary disadvantages associated with ethane and pentane analyses are the sampling techniques which are required to handle gases and the problems associated with background amounts of hydrocarbons in the air.

For these analyses, the gas samples are collected in traps of activated charcoal or molecular sieves [68,69], gas-tight bags [70,71], gas-tight tubes [72,73] or gas-tight syringes [74]. Care is generally taken to flush the collection sampling apparatus with the sample as part of the collection process. For the collection of breath samples, care should also be taken to evaluate the source of air the subject is breathing so that background hydrocarbons can be minimized or, at the least, standardized. For example, one investigator flowed ethane-free air through an activated charcoal trap to minimize background hydrocarbons in experiments with rats in a metabolic cage [68]. In experiments with humans, subjects have been required to either breath purified air during the sample collection procedure [69] or required to breath the room air for at least 1 h prior to sampling [72]. Other investigators have collected a parallel room air sample to directly account for room air contamination [70].

The analyses have been carried out using gas chromatography with both flame ionization detection [71,72,74] and mass spectrometric detection [69–71]. Injection of samples is made either by direct injection of ml quantities of gas through a gas sampling valve [68,72,74] or by thermal desorption of traps such as cryotraps [69,71] or adsorbents [70]. The traps provide enhanced

sensitivity because they allow the analysis of larger amounts of gas in a single injection without compromising chromatography. Both packed [68,74] and capillary columns [70–72] have been used. As seen in Fig. 7, the challenge of developing appropriate chromatographic conditions is the separation of the ethane and pentane from components such as ethanol, isoprene, and acetone that are found in some samples at significant levels. Recent reports have detailed the co-elution of pentane and isoprene under some experimental conditions leading to falsely high pentane values [71,72]. Therefore, although the majority of methods utilize flame ionization detection, mass spectrometric detection may be advantageous for the selective detection of pentane under conditions where isoprene is found to co-elute [71].

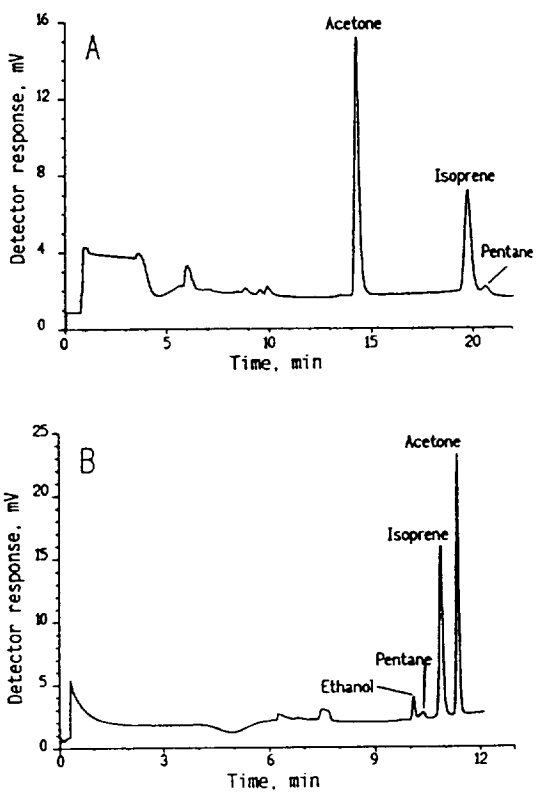

Fig. 7. GC analysis of hydrocarbons in human breath. Breath was collected in a polyvinylchloride tube and transferred to a 60-ml polyethylene syringe. The analyses were performed by capillary gas chromatography with flame ionization detection using either (A) a Poraplot Q capillary column or (B) a Poraplot U capillary column. (From Ref. [72], used with permission.).

3. Cholesterol oxidation products

Cholesterol is another lipid species found in significant abundance in biological systems that is able to participate in free radical initiated oxidation chemistry. Oxidation products indicative of hydrogen abstraction at the 17-, 20-, 22-, 23-, 24-, 25-, and 26-positions of cholesterol have all been detected [75]. However, the analytical methods to determine cholesterol oxidation have focused on the products of the primary hydrogen abstraction site, the allylic 7-position such as 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, along with selected other products of hydrogen abstraction at the 5- and 6-position such as 5α,6α- and 5β,6β-epoxycholesterol, and 5α-cholestane-3β,5α,6β-triol.

The methods for the determination of cholesterol oxidation products have relied on gas chromatography because of the variety of cholesterol oxidation products and the large amounts of cholesterol found in the samples. Capillary gas chromatography with flame ionization detection has been used in a series of experiments demonstrating the occurrence of cholesterol oxides in aortic tissue of experimental animals [76], the effectiveness of antioxidant therapy in reducing the formation of cholesterol oxides in this model [77], and the presence of cholesterol oxides in circulating, oxidized LDL in monkeys [78]. One such analysis using a 30-m DB-1 capillary column programmed from 250°C to 290°C with flame ionization detection is shown in Fig. 8. In this protocol, the lipids were extracted with a chloroform–methanol mixture containing antioxidants, fractionated by solid-phase extraction and converted to the trimethylsilyl ethers for analysis. Quantitation of 7α- and 7β-hydroxycholesterol, 5α,6α- and 5β,6β-epoxycholesterol, 5α-cholestane-3β,5α,6β-triol, 7-ketocholesterol, and 25-hydroxycholesterol was achieved using 5α-cholestane as the internal standard. Other investigators

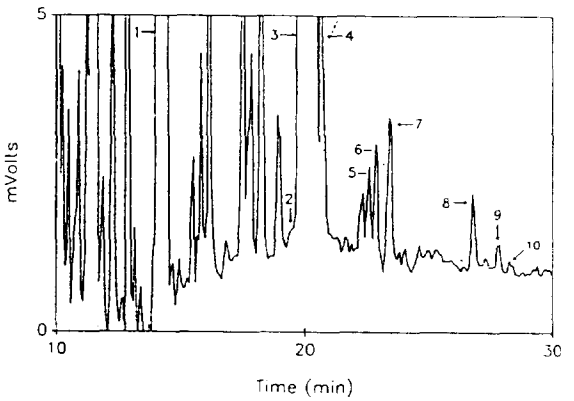

Fig. 8. GC analysis of cholesterol oxidation products in aortic tissue. 7α-Hydroxycholesterol (peak 2), cholesterol (peak 3), 3,5-cholesteroldiene (peak 4), 7β-hydroxycholesterol (peak 5), β-epoxycholesterol (peak 6), α-epoxycholesterol (peak 7), 5α-cholestane-3β,5α,6β-triol (peak 8), 7-ketocholesterol (peak 9), and 25-hydroxycholesterol (peak 10) were detected as their trimethylsilyl ether derivatives using a 30-m DB1 capillary column with flame ionization detection. 5α-Cholestane (peak 1) was used as an internal standard. (From Ref. [76], used with permission.).

have used ether extraction with the formation of trimethylsilyl ether derivatives for the gas chromatographic analysis of a similar series of cholesterol oxidation products in heart tissues [79].

Methods based on GC–MS analyses have also been described for cholesterol oxidation products. For example, analysis of red cell membranes by GC–MS has found that sickle red cells contain greater amounts of 5α,6α-epoxycholesterol, 5α-cholestane-3β,5α,6β-triol, 7-ketocholesterol, and 19-hydroxycholesterol [80]. These experiments used cholesterol butyrate as an internal standard and thin layer chromatography to fractionate the cholesterol and cholesterol oxides prior to analysis. Other investigators have taken advantage of GC–MS analyses to add a stable isotope monitor of adventitious cholesterol oxidation in the sample preparation process [81]. [$^2H_9$]Cholesterol is added at the beginning of the preparation protocol, and the mass spectrometric analysis used to simultaneously monitor for both [$^2H_9$]-labeled and unlabeled oxidation products. This analysis uses trimethylsilyl derivatives for positive ion chemical ionization analysis and heptafluorobutyryl derivatives for negative ion chemical ionization analysis. The authors identify a sample preparation protocol that includes purging O_2 from solvents, collecting fractions on ice, evaporating solvents at room temperature, and handling the samples in reduced light that minimizes cholesterol oxidation in sample processing.

4. Conclusion

As one evaluates the analytical methods described in this review, two general impressions are made of their utility in investigations of oxidative stress. First, the methods available for the analysis of lipid oxidation products are diverse in terms of both the types of analytes, and the analytical technologies applied. This diversity is important in that it provides a variety of approaches to evaluations of oxidative stress in biological systems. Second, all of the methods are being continually perfected and evaluated, despite as much as two decades of work with compounds like malondialdehyde. It is important that the analytical technologies used to determine lipid oxidation products continue to respond to the demands of the biological investigators to solve additional methodological problems that will arise. As one evaluates these techniques, however, it is also clear that new approaches to monitoring oxidative stress are needed. These new approaches can take the form of methods for new analytes as exemplified by the methods for F_2-isoprostanes and 4-hydroxy-2-nonenal. Interest, however, must also begin to turn to methods for metabolites of all of these compounds. One might argue that reliance on a simplistic model quantifying only one pool of a biologically active species that possesses a complex metabolic cascade might be misleading rather than informative since quantitative changes in that pool can be due to changes in both rate of production and/or rate of metabolism.

References

[1] D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo and J.L. Witzum, *New Engl. J. Med.*, 320 (1989) 915.

[2] H.-X. Deng, A. Hentati, J.A. Tainer, Z. Iqbal, A. Cayabyab, W.-Y. Hung, E.D. Getzoff, P. Hu, B. Herzfeldt, R.P. Roos, C. Warner, G. Deng, E. Soriano, C. Smyth, H.E. Parge, A. Ahmed, A.D. Roses, R.A. Hallewell, M.A. Pericak-Vance and T. Siddique, *Science*, 261 (1993) 1047.
[3] E.R. Stadtman, *Science*, 257 (1992) 1220.
[4] F.P. Corongiu and S. Banni, *Methods Enzymol.*, 233 (1994) 303–310.
[5] W. Jessup, R.T. Dean and J.M. Gebicki, *Methods Enzymol.*, 233 (1994) 289–303.
[6] S.P. Wolff, *Methods Enzymol.*, 233 (1994) 182–189.
[7] C.Y. O'Gara, K.R. Maddipati and L.J. Marnett, *Chem. Res. Toxicol.*, 2 (1989) 295.
[8] Y. Yamamoto, B. Frei and B.N. Ames, *Methods Enzymol.*, 186 (1990) 371–380.
[9] A.E. Holley and T.F. Slater, *Free Rad. Res. Comm.*, 15 (1991) 51.
[10] Y. Yamamoto, *Methods Enzymol.*, 233 (1994) 319–324.
[11] T. Miyazawa, K. Fujimoto and S. Oikawa, *Biomed. Chromatogr.*, 4 (1990) 131.
[12] T. Miyazawa, T. Suzuki, K. Fujimoto and T. Kaneda, *J. Biochem.*, 107 (1990) 689.
[13] T. Miyazawa, K. Fujimoto, T. Suzuki and K. Yasuda, *Methods Enzymol.*, 233 (1994) 324–332.
[14] T. Miyazawa, T. Suzuki, K. Fujimoto and K. Yasuda, *J. Lipid Res.*, 33 (1992) 1051.
[15] H.J. Helbock, P.A. Motchnik and B.N. Ames, *Pediatrics*, 91 (1993) 83.
[16] K. Akasaka, S. Ijichi, K. Watanabe, H. Ohrui and H. Meguro, *J. Chromatogr.*, 596 (1992) 197.
[17] K. Akasaka, H. Ohrui, H. Meguro and M. Tamura, *J. Chromatogr.*, 617 (1993) 205.
[18] K. Akasaka, H. Ohrui and H. Meguro, *J. Chromatogr.*, 622 (1993) 153.
[19] G.R. DeMeyer, H. Bult and A.G. Herman, *Biochem. Pharmacol.*, 42 (1991) 279.
[20] C.E. Thomas and R.L. Jackson, *J. Pharmacol. Exper. Therapeut.*, 256 (1991) 1182.
[21] D.W. Thomas, F.J.G.M. van Kuijk, E.A. Dratz and R.J. Stephens, *Anal. Biochem.*, 198 (1991) 104.
[22] D.W. Thomas, F.J.G.M. van Kuijk and R.J. Stephens, *Anal. Biochem.*, 206 (1992) 353.
[23] D.M. Guido, R. McKenna and W.R. Mathews, *Anal. Biochem.*, 209 (1993) 123.
[24] S.B. Turnipseed, A.J. Allentoff and J.A. Thompson, *Anal. Biochem.*, 213 (1993) 218.
[25] J.M. Petruska, K.O. Leslie and B.T. Mossman, *Free Rad. Biol. Med.*, 11 (1991) 425.
[26] I.S. Young and E.R. Trimble, *Ann. Clin. Biochem.*, 28 (1991) 504.
[27] M.J. Richard, P. Guiraud, J. Meo and A. Favier, *J. Chromatogr.*, 577 (1992) 9.
[28] H. Rabl, G. Khoschsorur, T. Colombo, F. Tatzber and H. Esterbauer, *Free Rad. Biol. Med.*, 13 (1992) 281.
[29] H. Kosugi, T. Kojima and K. Kikugawa, *Lipids*, 28 (1993) 337.
[30] I.S. Young, J.A. Purvis, J.H. Lightbody, A.A. Adgey and E.R. Trimble, *Eur. Heart J.*, 14 (1993) 1027.
[31] C. Ceconi, A. Cargnoni, E. Pasini, E. Condorelli, S. Curello and R. Ferrari, *Am. J. Physiol.*, 260 (1991) H1057.
[32] K. Kanamaru, B.K. Weir, I. Simpson, T. Witbeck and M. Grace, *J. Neurosurg.*, 74 (1991) 454.
[33] W.A. Behrens and R. Madere, *Lipids*, 26 (1991) 232.
[34] E. Tani, E. Rekka and P.N. Kourounakis, *Pharmazie*, 46 (1991) 118.
[35] H.S. Lee, D.W. Shoeman and A.S. Csallany, *Lipids*, 27 (1992) 124.
[36] P. Cogrel, I. Morel, G. Lescoat, M. Chevanne, P. Brissot, P. Cillard and J. Cillard, *Lipids*, 28 (1993) 115.
[37] G.A. Cordis, N. Maulik, D. Bagchi, R.M. Engelman and D.K. Das, *J. Chromatogr.*, 632 (1993) 97.
[38] D. Bagchi, M. Bagchi, E.A. Hassoun and S.J. Stohs, *J. Anal. Toxicol.*, 17 (1993) 411.
[39] D. Bagchi, M. Bagchi, E. Hassoun, J. Moser and S.J. Stohs, *J. Biochem. Toxicol.*, 8 (1993) 101.
[40] D. Bagchi, M. Bagchi, E. Hassounand S.J. Stohs, *Pharmacology*, 47 (1993) 209.
[41] C. Des Rosiers, M.J. Rivest, M.J. Boily, M. Jette, A. Carrobe-Cohen and A. Kumar, *Anal. Biochem.*, 208 (1993) 161.
[42] K.J. Dennis and T. Shibamoto, *Lipids*, 25 (1990) 460.
[43] H. Tamura and T. Shibamoto, *Lipids*, 26 (1991) 170.
[44] D. Bagchi, M. Bagchi, E. Hassoun and S.J. Stohs, *Toxicology*, 75 (1992) 81.
[45] D. Bagchi, M.A. Shara, M. Bagchi, E.A. Hassoun and S.J. Stohs, *Toxicol. Appl. Pharmacol.*, 123 (1993) 83.
[46] G.A. Cordis, D. Bagchi, N. Maulik and D.K. Das, *J. Chromatogr. A*, 661 (1994) 181.
[47] M.A. Shara, P.H. Dickson, D. Bagchi and S.J. Stohs, *J. Chromatogr.*, 576 (1992) 221.
[48] T. Grune, W. Siems, A. Werner, G. Gerber, M. Kostic and H. Esterbauer, *J. Chromatogr.*, 520 (1990) 411.
[49] C. Goldring, A.F Casini, E. Maellaro, B. Del Bello and M. Comporti, *Lipids*, 28 (1993) 141.
[50] T. Grune, W.G. Siems, J. Kowalewski and H. Esterbauer, *Life Sciences*, 55 (1994) 693.
[51] J.K. Beckman, S.A. Morley Jr. and H.L. Greene, *Lipids*, 26 (1991) 155.
[52] G. Bringmann, M. Gassen and S. Schneider, *J. Chromatogr. A*, 670 (1994) 153.
[53] A.D. Jones, C.K. Winter, M.H. Bounarati and H.J. Segall, *Biol. Mass Spectrom.*, 22 (1993) 68.
[54] M. Kinter, S. Sullivan, R.J. Roberts and D. Spitz, *J. Chromatogr.*, 578 (1992) 9.
[55] C. Norsten-Hoog and T. Cronholm, *Anal. Biochem.*, 189 (1990) 131.
[56] W.J. Blanchflower, D.M. Walsh, S. Kennedy and D.G. Kennedy, *Lipids*, 28 (1993) 261.
[57] K. Uchida and E.R. Stadtman, *Methods Enzymol.*, 233 (1994) 371–380.
[58] N.A. Porter and M.O. Funk, *J. Org. Chem.*, 40 (1975) 3614.

[59] J.D. Morrow, K.E. Hill, R.F. Burk, T.M. Nammour, K.F. Badr and L.J. Roberts II, *Proc. Natl. Acad. Sci. USA*, 87 (1990) 9383.
[60] J.D. Morrow, T.M. Harris, L.J. Roberts II, *Anal. Biochem.*, 184 (1990) 1.
[61] J.D. Morrow and L.J. Roberts II, *Free Rad. Biol. Med.*, 10 (1991) 195.
[62] J.A. Awad, J.D. Morrow, K. Takahashi and L.J. Roberts II, *J. Biol. Chem.*, 268 (1993) 4161.
[63] S.M. Lynch, J.D. Morrow, L.J. Roberts and B. Frei, *J. Clin. Invest.*, 93 (1994) 998.
[64] N.K. Gopaul, J. Nourooz-Zadeh, A.I. Mallet and E.E. Änggård, *Biochem. Biophys. Res. Commun.*, 200 (1994) 338.
[65] J.D. Morrow, J.A. Awad, H.J. Boss, I.A. Blair and L.J. Roberts II, *Proc. Natl. Acad. Sci. USA*, 89 (1992) 10721.
[66] A.J. Dabbagh, T. Mannion, S.M Lynch and B. Frei, *Biochem. J.*, 300 (1994) 799.
[67] A.W. Longmire, L.L. Swift, L.J. Roberts, J.A. Awad, R.F. Burk and J.D. Morrow, *Biochem. Pharmacol.*, 47 (1994) 1173.
[68] J.T. Saari, F.D. Dickerson and M.P. Habib, *Proc. Soc. Exp. Biol. Med.*, 95 (1990) 30.
[69] M. Phillips, M. Sabas and J. Greenberg, *J. Clin. Path.*, 46 (1993) 861.
[70] A. Cailleux and P. Allain, *Free Rad. Res. Comm.*, 18 (1993) 323.
[71] D. Kohlmuller and W. Kochen, *Anal. Biochem.*, 210 (1993) 268.
[72] S. Mendis, P.A. Sobotka and D.E. Euler, *Clin. Chem.*, 40 (1994) 1485.
[73] P.A. Sobotka, D.K. Gupta, D.M. Lansky, M.R. Costanzo and E.J. Zarling, *J. Heart Lung Transplant.*, 13 (1994) 224.
[74] D. Ondrula, R.L. Nelson, G. Andrianopoulos, D. Schwartz, H. Abcarian, A. Birnbaum and J. Skosey, *Dis. Colon Rectum*, 36 (1993) 457.
[75] L.L. Smith, in C. Vigo-Pelfrey (Editor), *Membrane Lipid Oxidation, Vol. 1*, CRC Press, Boca Raton, 1990, Ch. 6, pp. 129–154.
[76] H.N. Hodis, D.W. Crawford and A. Sevanian, *Atherosclerosis*, 89 (1991) 117.
[77] H.N. Hodis, A. Chauhan, S. Hashimoto, D.W. Crawford and A. Sevanian, *Atherosclerosis*, 96 (1992) 125.
[78] H.N. Hodis, D.M. Kramsch, P. Avogaro, G. Bittolo-Bon, G. Cazzolato, J. Hwang, H. Peterson and A. Sevanian, *J. Lipid Res.*, 35 (1994) 669.
[79] H. Gangopadhyay, G.A. Cordis, R. Jones, X. Liu and D.K. Das, *J. Pharm. Biomed. Analysis*, 9 (1991) 1003.
[80] O. Kucuk, L.J. Lis, T. Dey, R. Mata, M.P. Westerman, S. Yachnin, R. Szostek, D. Tracy, J.W. Kauffman, D.A. Gage and C.C. Sweeley, *Biochim. Biophys. Acta*, 1103 (1992) 296.
[81] B.A. Wasilchuk, P.W. Le Quesne and P. Vouros, *Anal. Chem.*, 64 (1992) 1077.