Lipidomics in triacylglycerol and cholesteryl ester oxidation

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1. ABSTRACT

Although direct mass spectrometry is capable of identification the major molecular species of lipids in crude total lipid extracts, prior chromatographic isolation is necessary for detection and identification of the minor components. This is especially important for the analysis of the oxolipids, which usually occur in trace amounts in the total lipid extract, and require prior isolation for detailed analysis. Both thin-layer chromatography and adsorption cartridges provide effective means for isolation and enrichment of lipid classes, while gas-liquid chromatography and high performance liquid chromatography with on-line mass spectrometry permit further separation and identification of molecular species. Prior chromatographic resolution is absolutely necessary for the identification of isobaric and chiral molecules, which mass spectrometry/mass spectrometry (MS/MS) cannot distinguish. Both gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry applications may require the preparation of derivatives in order to improve the chromatographic and mass spectrometric properties of the oxolipids which is a small inconvenience for securing analytical reliability. The following chapter reviews the advantages and necessity of combined chromatographic-mass spectrometric approaches to successful identification and quantification of molecular species of oxoacylglycerols and o xocholesteryl esters in \textit{in-vitro} model studies of lipid peroxidation and in the analyses of oxolipids recovered from tissues.
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2. INTRODUCTION

Recent progress in proteomics and drug metabolism has identified lipidomics as an area in need for sensitive detection and detailed identification of low molecular weight ligands of various lipid binding proteins and membrane receptors. Lipidomics has been specifically defined as the full characterization of lipid molecular species and of their roles in expression of proteins involved in lipid metabolism and function, including gene regulation (1,2). Although direct MS/MS is capable of identification of the major molecular species of lipids in crude total lipid extracts, prior chromatographic isolation and enrichment is necessary for detection and identification of the minor components (3). This is especially important for the identification and structural characterization of isobaric, regioisomeric and enantiomeric isomers of the oxolipids (4,5). Non-polar capillary gas liquid chromatography (GC) in combination with mass spectrometry (MS) provided the definitive early methodology and remains indispensable for the analysis of oxofatty acids and oxosterols. For many other applications normal phase and reversed phase high performance liquid chromatography (HPLC) with on-line MS (LC-MS) has substituted for GC/MS to allow the separation and identification of the subclasses of oxolipids including oxocholesterol esters and oxotriacylglycerols, as well as their chemical derivatives (6), while chiral phase HPLC in combination with on-line MS permits the separation and identification of regional, reverse, and enantio isomers of oxofatty acids (7) and glycerolipids (8) following preparation of the dimitrophenyletheranes (DNPU), if necessary. When dealing with especially complex mixtures of minor components, both GLC and HPLC separation steps may be profitably preceded by preconcentration of the components, also chromatographic steps may be replaced by chemical derivatization (e. g. dinitrophenylhydrazine) (9), which modify the MS properties of the compounds of interest to permit identification without isolation, or by resorting to such specialized MS techniques as multiple reaction monitoring (MRM) (10) or coordinated ion spray (CIS) (11). In all instances the chromatographic steps provide in addition to preconcentration of the components, also chromatographic retention characteristics, that are critical for the identification of isobaric, isomeric and enantiomeric molecular species, which are not otherwise resolved by MS/MS.

In the following chapter, selected studies have been reviewed to illustrate the advantages of chromatographic prefracionation in MS identification of the molecular species of various neutral oxolipids derived from acylglycerols and cholesteryl esters. The discussion is preceded with a brief review of the formation and identification of unesterified oxofatty acids and sterols.

3. EXPERIMENTAL STRATEGY

Since oxolipids are formed by mere exposure to air, a critical methodological issue is the use of techniques that prevent significant artifact formation by oxidation during sample preparation, and during chromatography, which may be used for sample purification. Early workers attributed the loss of polyunsaturated fatty acids (FA) on TLC to iodination during staining with iodine vapor, while later researchers recognized the general oxidative hazards of TLC itself. Lipid peroxidation on chromatographic columns, however, has become recognized only recently (12,13) and has led to the recommendation to eliminate the chromatographic step in tandem MS/MS analyses (14).

3.1. Preparation of sample extracts

In order to minimize autoxidation of unsaturated lipids during isolation, it is necessary to exclude air from the extraction solvents, and transition metals from chromatographic adsorbents. Thus may be accomplished by conducting the extraction with inert solvents (CHCl₃/MeOH, but not Et₂O, which readily forms hydroperoxides) under inert gas (e. g., oxygen-free N₂, Ar) or in presence of appropriate antioxidants (e. g., butylated hydroxytoluene, BHT) the adsorbents for TLC and column cartridges must be washed with chelating agents to remove divalent ions such as Cu²⁺ and Fe²⁺. Podrez et al (12) recommends treating all aqueous solvents with Chelex-100 prior to use. Furthermore, all columns and filters are to be pre-rinsed with diethylenetriaminepentaaetic (DTPA)-containing solvents (at neutral pH). The washing may be done in-situ, at the normal flow rate of the column. Kenar et al. (15) recommend storing phosphate-buffered saline (PBS, pH 7.4, 50 mM) over Chelex-100 at least 24 h to remove transition metal contaminants. It is also good practice to complete the extraction as quickly as possible and, if necessary, to store the extracts at as low a temperature as possible (e. g. -80 °C). It may of interest to both Ag⁺-TLC and Ag⁺-HPLC that silver ions readily form coordination complexes, which greatly increase the sensitivity and accuracy of mass spectrometric analysis of non-polar components in complex lipid extracts (11,16, 17).

A detailed description of oxolipid isolation is provided by Podrez et al. (12). As an example, lipids are initially extracted three times by the method of Bligh and Dyer (18) from tissue homogenates (generated using a ratio of 1 g tissue: 10 ml 50 mM sodium phosphate (pH 7.0) supplemented with 2 mM DTPA or 100 µM BHT under Ar atmosphere. The combined extracts are rapidly dried under N₂, resuspended in MeOH/H₂O (98:2, v/v), and diluted with a known amount of internal standard, which also prevents losses on absorbptive surfaces and vessel walls. The neutral lipids in the lipid extracts are removed by passage through a C₁₈ minicolumn (Supelcolean LC-18 SPE tubes, 3 mL). The polar lipids are recovered by washing the column with MeOH. The neutral and polar lipid fractions are separately dried under N₂ and stored in the dark under an Ar atmosphere at -80 °C until analysis within 24 h. The overall recovery of each synthetic lipid (when analyzed at the trace levels observed in biological samples) has been confirmed to be in excess of 80% under the above conditions.

In other instances, tolerable recoveries of oxolipids have been obtained using the Folch et al. (19)
method of extraction under inert atmosphere (glove box) and in the presence of BHT. The synthetic antioxidant S20478 (50 μM) was capable of inhibiting the initiation and propagation of copper-mediated low density lipoprotein (LDL) oxidation, as determined by the time- and dose-dependent inhibition of the formation of conjugated dienes and thiobarbituric acid-reactive substances (TBARS) and preserving cholesteryl esters in their native form for 24 h (20). Yasuda and Narita (21) extracted cholesteryl ester and phospholipid hydroperoxides quantitatively from human plasma with a mixture of hexane/EtAc (1:1, v/v), while Mashima et al. (22) reported the extraction of cholesteryl linoleate hydroperoxides from normal human plasma using MeOH/hexane (1:7, v/v) supplemented with antioxidant, reductant, and an internal standard for a study of regioisomer distribution of cholesteryl linoleate hydroperoxides and hydroxides.

3.2. Preparation of derivatives

Although many oxolipids may be subjected to HPLC in the free form, a preparation of derivatives, where possible, is desirable for increased stability, ease of chromatographic properties. Harrison et al. (27) have described various microreactions for the derivatization of oxolipids and stable isotope labeled oxolipids are noted below.

The lipid hydroperoxides may be reduced to hydroxides by reaction with NaBH₄ (23) or triphenylphosphine (24), with the result that the more stable hydroxides may be further derivatized with trimethylsilyl (TMS) or tert-butyldimethylsilyl (tert-BDMS) chloride for specific identification by GC-MS (25). HPLC may be used for separation of the core aldehydes and ketones as the hydrazones prepared by reaction with 2,4-dinitrophenylhydrazine (DNPH) (9) or 1-methyl-DNPH (26). Unsaturated oxolipids may be subjected to hydrogenation or deuteration, which results in a specific molecular weight change, which may be accompanied by an alteration in the chromatographic properties. Harrison et al. (27) have described various microreactions for the derivatization of oxolipids, including preparation of TMS ethers and esters, and oxime derivatives. The ultimate derivatization results in the release of an oxofatty acid from the glycerolipid or cholesteryl ester molecule, which may be effected by enzymatic or chemical methods. However, the oxidation products of cholesteryl ester linoleate have been shown to be resistant to hydrolysis by macrophage cholesteryl ester hydrolase (28).

Increased sensitivity and specificity of MS detection is also obtained by analysis of the DNPU (29) and DNPH (30) of the lipid alcohols and carbonyls, respectively. Kuksis et al. (31) reported a 100 fold increase in the sensitivity (compared to detection by positive ionization with ammonia) of molecular species of triacylglycerols (TAGs) following formation of the pseudomolecular ions [M+Cl]⁻ as a result of inclusion 1% CH₃CH₂Cl in the HPLC mobile phase (10-90% linear gradient of CH₃CH₂CN in CH₃CN). The sensitivity of detection of the DNPU and DNPH derivatives compares to that of fluorescence detection of the corresponding hydroperoxides (32). Lee et al. (3) have demonstrated that electron capture (EC) APCI provides an increase in sensitivity of two orders of magnitude when compared with conventional negative ion APCI-MS methodology. Bioactive lipids are first derivatized with an electron-capturing group such as pentafluorobenzyl (PFB) moiety before LC analysis.

3.3. Preparation of standards

Pure standards of many unsaturated fatty acids, glycerol esters, cholesteryl esters and free sterols may be obtained from commercial suppliers. Oxolipid standards, however, must be prepared in the laboratory. Some outstanding preparations of oxolipids and stable isotope labeled oxolipids are noted below.

The preparation of 13-S-hydroperoxy-9-Z,11-E,15-Z-octadecadienoic acid (13-S-HPOD) and 13-S-hydroperoxy-9-Z,11-E15-Z-octadecatrienoic acid (13-S-HPOT) using soybean lipoxygenase (LOX) (Sigma, L-8383) has been described by Schneider et al. (34). Typically, 5 mg fatty acid was diluted in 10 ml of 0.1 M borate buffer (pH 9.0), flushed with oxygen at 0°C. The reaction was initiated by addition of 1 mg soybean LOX and the progress of the reaction monitored at 234 nm. Likewise, 9-S-hydroperoxy-10-E,12-Z-octadecadienoic acid (9-S-HPOD) and 9-S-hydroperoxy-10-E,12-Z,15-Z-octadecatrienoic acid (9-S-HPOT) were prepared using tomatolic LOX (34). Five mg fatty acid was dissolved in 10 ml 0.1 M phosphate buffer (pH 6.5) and flushed with oxygen at 0°C.

Two procedures have been described by Gardner and Grove (35) for the preparation of 9(S)-hydroperoxides of linoleic and linolenic acids by maize lipoxygenase. The authors state that this alternative method of preparing 9-hydroperoxides has advantages over other commonly used plant lipoxygenses.

Isomeric monohydroperoxides of methyl arachidonate and 2-linoleoyl-1,3-dipalmitoylglycerol have been obtained (36) by autoxidation (40 °C in the dark) in the presence of α-tocopherol (5%; 0.1 M), which suppresses the formation of trans,trans-isomers and secondary oxidation products (hydroperoxepipidoxides). Only cis,trans-conjugated diene monohydroperoxides are formed. Preparative normal phase HPLC was used to isolate the 2(13-hydroperoxy-9-cis,11-trans-
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octadecadienoyl)- and 2(9-hydroperoxy-10-trans,12-cis-octadecadienoyl)-1,3-dipalmitoylglycerols from the monohydroperoxide fraction. FAB-MS was used to characterize the hydroperoxide isomers. The spectra were virtually identical. In the positive ion mode [M+H]+ ions at m/z 863 were absent but ions corresponding to loss of both water (m/z 845) and H2O2 (m/z 829) were observed. Also supporting the hydroperoxide structure were ions corresponding to loss of a palmitic acid residue (m/z 607, [M+H-256]+) followed by losses of water (m/z 589) and hydrogen peroxide (m/z 573). 5,8,11,14-20:4 was produced from methyl 20:5n3 by 14(15)-epoxy-5,8,11,17-20:4, and arachidonate by Yin et al. (39) prepared vicinal diol standards of 5,8,11,14,17-eicosapentaenoic acid. A mixture of cis-5(6)-epoxy-8,11,14,17-20:4, cis-8(9)-epoxy-5,11,14,17-20:4, cis-11(12)-epoxy-5,8,14,17-20:4, cis-14(15)-epoxy-5,8,11,17-20:4, and cis-17(18)-epoxy-5,8,11,14-20:4 was produced from methyl 20:5n3 by reacting with m-chloroperbenzoic acid. Regioisomers were resolved by normal phase HPLC as follows: 14:15- (32.8); 11,12- (35.6 min); 17,18- (44.6 min), 8,9- (44.6 min), and 5,5-epoxy-20:4 (61.6 min). Each isomer, absorbing at 192 nm, was collected. The co-eluting 17,18- and 8,9-epoxy-20:4 regioisomers were resolved by further chromatography.

VanRollins and Murphy (40) have described the synthesis and characterization of all ten theoretically predicted rac monohydroxy isomers of docosahexaenoic acid. The HDE isomers were generated by autoxidation coupled to NaN3 reduction (see below). Kerwin et al. (41) prepared 15O fatty acid derivatives using porcine liver esterase in 3H2O. One or two 15O were introduced into the carboxyl moiety. Deuterated analogs were synthesized by dissolving and evaporating fatty acids (two to three times) in MeOD. The structure of the stable isotope labeled fatty acids or methyl esters was determined by mass spectrometry. Similar methods are suitable for the preparation of hydroperoxides and hydroxides from autoxidized TAGs (see above) and cholesteryl esters.

Kuklev et al. (42) described a convenient preparative method for the synthesis of hydroxydioenoic (9-HODE and 13-HODE) and ketodienoic compounds (9-KODE and 13-KODE) from natural linoleic acid. Methyl linoleate was treated with m-chloroperbenzoic acid in alcoholic solution, giving a mixture of monoepoxides, which upon treatment with HBr in MeOH yielded a mixture of bromohydrins. The bromohydrins were converted in high yield to a mixture of bromoketones, which debrominated by DBU to produce a mixture of ketodienoic acids, which upon reduction with KBH4 in MeOH led to the corresponding hydroxydienoic acid methyl esters. The keto- and hydroxyl fatty acids obtained were characterized by TLC, HPLC, NMR and MS.

Morrow and Roberts (43) prepared standard [3H4] PGF2alpha as an internal standard using a modification of the method described for quantification of F2-isops. Bernoud-Hubac et al. (44) reported the oxidation of 22:6n3 to the F2-neuroprostanes and neuroketals. The oxidation was performed with in vitro using iron/ADP/ascorbate.

Kozak et al. (45) prepared pentadeuterated by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl-mediated coupling of the target prostaglandin with anhydrous 2H5-glycerol, purified by preparative TLC, and characterized by MS.

Yin et al. (46) isolated a novel class of peroxides termed dioxygeno-isoprostanes having a bicyclic endoperoxide moiety characteristic of isoprostane and a dioxygeno peroxide functionality in the same molecule from in vitro oxidation cholesteryl arachidonate. The structure of these compounds was confirmed by independent synthesis from PGF2alpha.

Sjovall et al. (47) prepared synthetic oxotriacylglycerols (18:0/18:0/18:1-OOH, 18:0/18:1/18:0- OOH, 18:1/18:1/18:9-di-OOH, 18:0/18:0/18:2-di-OOH,
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18:0/18:0/18:2-OOH, 16:0/18:2/16:0-OOH, and 18:0/18:2/18:0-mono- and di-OOH) by reacting with tert-BOOH and by photosensitized oxidation with methylene blue under a photographer’s lamp (48). Hydroperoxides were reduced to the corresponding hydroxyTAGs by reacting with NaBH₄ or triphenylphosphine (23,24). The preparation of TAG epoxides (18:0/18:0/18:1-epoxy, 18:0/18:1/18:0-diepox, and 18:0/18:0/18:2-diepox) was achieved by reaction with 3-chloroperbenzoic acid. Specifically prepared were: 18:0/18:0/18:0 monoepoxides and 18:1/18:1/18:0 and 18:0/18:0/18:2 diepoxides.

Bauer-Plank and Steenhorst-Slikkerveer (49) prepared the hydroperoxides 1-palmitoyl-2-(13-hydroperoxy-linoleoyl)-3-stearoyl-glycerol and 1-palmitoyl-2-stearoyl-3-(13-hydroperoxy-linoleoyl)-glycerol by coupling a protected 13-hydroperoxylinoleic acid with the desired DAG. After synthesis the hydroperoxide was deprotected. The purity of the compounds was between 95 and 99% as determined by ¹H NMR.

Sjovall et al. (47) prepared the triacylglycerol core aldehydes (18:0/18:0/18:0, 18:0/18:0:18:0, 18:0/18:1/18:0 by reduction of ozonides with triphenylphosphine as described by Ravandi et al. (50). The aldehydes were converted into the corresponding DNPH derivatives by reaction with DNP hydrazine as described by Esterbauer et al. (9). All the above synthetic intermediates and final products were purified by appropriate TLC systems to provide milligrams quantities of reference standards.

A synthetic method of preparation of TAG core aldehydes has been proposed by Mittelbach et al. (51). The method starts with the ozonization of oleic acid and further reduction with dimethylsulfide to yield 9,9-dimethoxynonanoic acid. The condensation of the dimethoxynoanoic acid with glycerol and further condensation with fatty acids in presence of N,N'-dicyclohexylcarbodiimide yields 49-52% of TAG core aldehyde. The reaction product was characterized by ¹H NMR and GC-MS. The EI mass spectrum yielded [M-FA+] as a major ion. This method of aldehyde preparation can be used to synthesize core aldehydes which still contain unsaturated residues.

Several oxocholesterols are available from commercial sources: 5-cholesten-3-beta-ol-7-one; 5-cholestan-5beta,6beta-epoxy-3beta-ol, 5-cholestone. 25-Hydroxycholesterol, cholestane-triol, 7beta-hydroxycholesterol, 5alpha-epoxycholesten-3beta,19-diol; 5-cholesten-3beta,20alpha-diol; and cholestane-3beta,5alpha,6beta-triol, 20alpha-hydroxycholesterol, 6-ketocholesterol, 19-hydroxycholesterol as well as cholesterol (Sigma Chemical Co., St. Louis, MO); The standards 5-cholesten-3beta,25-diol (25-OH), 5-cholesten-3beta,4beta-diol (4beta-OH), 5-cholesten-3beta,7alpha-diol (7alpha-OH) and 5-cholesten-3beta,7beta-diol (7beta-OH) (Steraloids, Inc., Wilton, NH); 7alpha-hydroxycholesterol, 5beta-epoxycholesterol; 4,6-cholestadien-3-one; 3,5-cholestadien-7-one and 5alpha-cholestan-3,6-dione (Steraloids, Inc., Wilton, NH). These oxosterol preparations have been tested for purity and identity by normal phase and reversed phase HPLC with MS (52, 53).

Malavasi et al. (54) have reported the preparation of authentic 7alpha-hydroperoxy and 7alpha-hydroxycholesterol, 7-ketocholesterol and 7beta-hydroxycholesterol according to established procedures. Dzelotovic et al. (55) reported the synthesis of authentic 7alpha-hydroperoxide of cholesterol by isomerization of 5alpha-hydroxycholesterol-7-en-3beta-ol according established procedures. Dzelotovic et al. (56) also reported the preparation of deuterated oxocholesterol standards using established chemical methods: [26,26,26,27,27,27-²H₃]5,6alpha-Epoxy-5alpha-cholestan-3beta-oI, [26,26,26,27,27,27-²H₃]5α-Cholest-5-ene-3beta-oI, [26,26,26,27,27,27-²H₃]5α-Cholest-5-ene-3beta-oI; [26,26,26,27,27,27-²H₃]5α-Cholest-5-ene-3beta-oI; [26,26,26,27,27,27-²H₃]5α-Cholest-5-ene-3beta-oI.

Kenar et al. (15) prepared oxocholesterol linoleates by autoxidation and used HPLC to separate and isolate four hydroperoxides, which were characterized by analytical HPLC, mass spectrometry and NMR. The hydroperoxides (cholesterol 13-hydroperoxyoctadecac-acis-9,trans-11-dienoate, cholesteryl 13-hydroperoxyoctadecac-trans-9, trans-11-dienoate, mixture of cholesteryl 9-hydroperoxyoctadecac-trans-10, cis-12-dienoate and cholesteryl 9-hydroperoxyoctadecac-trans-10, trans-12-dienoate) were converted into the corresponding cholesteryl linolate hydroperoxides by reduction with triphenylphosphine and the products resolved by preparative HPLC (cholesterol 13-hydroperoxyoctadecac-acis-9,trans-11-dienoate; cholesteryl 13-hydroperoxyoctadecac-trans-9,trans-11-dienoate; cholesteryl 9-hydroperoxyoctadecac-trans-10, cis-12-dienoate and cholesteryl 9-hydroperoxyoctadecac-trans-10, trans-12-dienoate).

Mashima et al. (22) prepared standard regioisomeric cholesteryl linolate hydroperoxides and hydroxides as follows. Cholesteryl 18:2-OOH was prepared by spontaneous autoxidation of hydroperoxide-free cholesterol 18:2 (80 mol) in 1 ml of hexane at room temperature for 10 days. Addition of triphenylphosphine reduced cholesteryl 18:2-OOH to cholesteryl 18:2-OH. The cholesteryl 18:2 hydroperoxides and hydroxides were resolved by semipreparative silica gel column using hexane-2-propanol (1000:5) into the four regioisomers (15). Cholesteryl 20:2 hydroperoxides and oxides were prepared similarly. Cholesteryl 15-hydroxy-11Z,13E-eicosadienoate (15Z-Echo20:2-OH), cholesteryl 15-hydroxy-11E,13E-eicosadienoate (15E-Echo20:2-OH), cholesteryl 11-hydroxy-12E,14Z-eicosadienoate (11EZ-Ch20:2-OH), and cholesteryl 11-hydroxy-12E,14Z-eicosadienoate (11EE-Ch20:2-OH) were separated by using an identical, normal phase HPLC procedure.

Cholesteryl 18:3 was oxidized in hexane in the presence of 5 mol % of alpha-tocopherol at room
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temperature for 23 days, alpha-Tocopherol was added to decrease the formation of the ZEE isomers of cholesteryl 18:3-hydroperoxides (Ch18:3-OOH) and to accelerate autoxidation (36). Amongst the four ZEE isomers of Ch 18:3 hydroxides, cholesteryl 6-hydroxy-7E,9Z,12Z-octadecatrienoate was selected as an internal standard.

Plat et al. (57) prepared synthetic oxophytosterols from a commercial mixture of plant sterols containing 60% sirosterol (Sit), 36% campsterol (Camp) and 3% stigmasterol using the methods described for oxygenation of cholesterol by Li et al. (58). A preparation of 7-hydroxyphytosterols was shown by GC/MS to contain a mixture of 7alpha-1OH-Sit, 7alpha-1OH-Camp, 7beta-1OH-Sit, and 7beta-1OH-Camp and traces of 7-OH stigmasterol. The 7-Keto plant sterol preparation was shown to contain a mixture of 7-keto-Sit, 7-keto-Camp. The preparation of 5,6-epoxy plant sterols contained 5alpha,6alpha-epoxy-Sit, 5alpha,6alpha-epoxy-Camp, 5beta,6beta-epoxy-Sit, and 5beta,6beta-epoxy-Camp. The preparation of the plant sterol triols contained a mixture of 3beta,5alpha,6beta-trihydroxy-Sit and 3beta,5alpha,6alpha-trihydroxy-Sit. In addition, [2,2,4,4,6-2H5]3beta,5alpha,6beta-trihydroxy-Phytosterol-7 and [2,2,4,4,6-2H5]3beta,5beta,6beta-trihydroxy-Phytosterol were prepared as internal standards. The deuterated oxosterols were prepared from the corresponding deuterated plant sterols purchased from Medical Isotopes Inc., Pelham, USA. The authors provided isotope differentiation (corrected for natural background of 13C). Because d4-trihydroxy-Sit was the most abundant isotope, m/z 488 for d4-tri-hydroxy-Sit was used as internal standard. In case of the deuterated 7-keto standards, the most abundant d7-7-keto-Sit (m/z 504).

Yin et al. (38) have described the oxidation of cholesteryl arachidonate to primary hydroperoxides using methyl tert-butyldimethylchroman-2-carboxylate and di-tert-butyl hyponitrite as the oxidizing agents. The reaction was stirred under oxygen at 37 °C for 24 h, quenched with BHT, and the seven major fractions obtained separated by analytical HPLC and the 15-hydroperoxyicosatetraenoic acid (HPETE) (13.80 min), 12-HPETE (14.54 min), 11-HPETE (16.11 min), 9-HPETE (20.91 min), 8-HPETE (21.29 min) and 5-HPETE (26.11 min) collected and purified. The compounds were characterized by LC/MS and after reduction by GC/NICI MS.

Kamido et al. (25) prepared cholesteryl [5-oxo]valerate and cholesteryl [9-oxo]nonanoate from cholesteryl arachidonate and cholesteryl oleate, respectively, by osmium tetroxide oxidation and carbon-carbon bond cleavage with periodic acid. The core aldehydes were characterized by GC-MS with ammonia. Later, Bochzel et al. (60) described a new route for the preparation of cholesteryl core aldehydes. It starts with the ozonization of oleic acid, and further reduction with dimethyl sulfide to yield either 9,9-dimethoxynonanoic acid. The condensation of the 9,9-dimethoxynonanoic acid with cholesterol is achieved with N,N'-dicyclohexylcarbodimide in dichloromethane. The reference core aldehydes are best employed as the DNPH derivatives, which possess excellent chromatographic and MS properties (61).

3.4. Analytical procedures
3.4.1. TLC separation and isolation of oxolipids
Both conventional and specialized chromatography techniques are utilized for the segregation of lipid classes based on polarity, as well as for the enrichment of minor oxolipid components from total lipid extracts. TLC is usually performed on conventional analytical plates (20 x 20 cm covered with a 250 µ thick layer of silica gel) and a variety of organic solvents appropriate for each lipid class (see text for selected applications). In specific instances borate may be incorporated into the silica gel in order to control the isomerization of the oxolipids, while in other cases silver nitrate may be added to the silica gel to bring about a resolution of saturated and unsaturated components. The resolved lipid fractions are located by spraying the plate with fluorescent dyes and viewing under UV light, and the lipid fractions recovered. If desired, the lipid fraction may be catalytically reduced (triphenylphosphine, NaBH4, NaCNBH3) or chemically derivatized (dinitrophenylhydrazone, dinitrophenylurethane or acetic anhydride) at this stage. High performance TLC (HPTLC) is carried out on microplates (see below for specific applications).

3.4.2. GLC separation of oxolipids
GLC separations of the volatile oxolipids are now performed with capillary columns of various lengths coated with non-polar liquid phases and helium as carrier gas. For this purpose the polar groups of oxolipids are converted into stable derivatives by NaBH4 reduction or hydrogenation and the new polar groups are converted into TMS or tert-BDMS ethers or esters. The progress of the separations is monitored by flame ionization detection, which also provides excellent quantification of eluents. The mass spectrometric detection is commonly achieved in the EI mode, but CI mode can be used with hydrogen as the carrier gas. GC/EI-MS is performed on-line with mass spectrometer serving as a detector for separations performed with capillary columns. Both positive and negative ionization modes may be utilized. Of special interest to lipodomics is the use of derivatives or adducts with electron capture properties, which increase greatly the sensitivity of detection of minor components. GLC separations have also been successfully performed with chiral phase columns, which have resolved both regioisomers and enantiomers.

3.4.3. HPLC separation of oxolipids
HPLC separations of oxolipids are performed using both normal and reversed phase columns with a variety of eluting solvents. The separations are monitored by UV and fluorescence or ELSD. HPLC provides characteristic order of elution of the solutes along with characteristic retention times, which are helpful in identification of isomeric and isobaric oxolipids. Of special interest is the separation of enantiomeric oxolipids using chiral phase HPLC (see below).
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LC-MS/MS is commonly performed with all liquid chromatographic systems coupled on-line with mass spectrometers of various configurations, including ion trap and time of flight instruments. While ESI and APCI are most commonly employed, other methods of ionization, such as CI, TSI, CSI and others are also used (see text for specific details). Both positive and negative ion modes are available and are utilized depending on the nature of the lipid or the derivative prepared. The eluting solvents may be selected to achieve a specific type of ionization, or a post-column addition of a non-resolving solvent may be made to enhance the ionization (see text for details of specific applications). In case of a single quadrupole instrument, two injections at different ionization voltages may be used (LC/ESI/CID-MS) to effect appropriate fragmentation of the detected molecules (see text for details of specific applications).

3.4.4. MS/MS

Despite the advantages of on-line GC-MS or LC-MS, direct MS/MS analysis of oxolipids has its own merits, especially, when the oxolipid fractions have been previously isolated by chromatography. This method permits the injection of larger amounts of sample than may be available from a GLC or HPLC separation. Direct MS/MS also permits work with underivatized samples that are unstable or not readily resolved by chromatography. Of great interest is the success of the MRM-MS and CIS-MS for the identification of oxolipids in complex mixtures of natural extracts (see below). In addition, modern MS/MS instruments are capable of different scanning modes, which may be advantageously applied to the detection and identification of minor lipid components in complex mixtures.

4. LIPIDOMICS OF OXOLIPIDS

Effective analysis of o xoacylglycerols and oxosterol esters requires the knowledge of the TLC, GLC, HPLC and MS behavior of the oxofatty acid and oxosterol moieties of the oxolipids as well as the structure of the known oxolipids. In the following a brief description is provided of the chromatographic separation and molecular structures of known oxofatty acids and oxosterols. It is both convenient and instructive to consider the oxolipid formation under primary and secondary products, although in an oxidation mixture both types of products may occur together. The discussion opens with a detailed consideration of the peroxidation products of free fatty acids or their methyl esters, although not all free fatty acid peroxidation products have thus far been identified in the glyceryl and cholesteryl ester forms. Likewise, the peroxides of free cholesterol are discussed prior to consideration of the ring oxides in cholesteryl esters.

4.1. Oxofatty acids

The hydroperoxides, hydroxides and epoxides of fatty acids considered in this review are all derivatives of unsaturated fatty acids and have been obtained by enzymatic or chemical oxidation and autoxidation, and may occur naturally in the free or esterified form. The products of fatty acid oxidation have been investigated most extensively and provide reference structures for the more complex glyceryl and cholesteryl esters to be discussed below.

4.1.1. Primary products

The exact mechanism of the oxofatty acid formation is not known with certainty, but plausible hypotheses, although not without challenge (62), have been advanced for both enzymatic (63) and chemical (64,65) oxidation to account for many of the final structures that have been isolated and identified beyond doubt by chromatographic, NMR and MS methods. Thus, autoxidation of methyl oleate yields 6 regiosomeric hydroperoxides (66), which are partially resolved by normal phase HPLC. The hydroperoxides were characterized by HPLC with CI-MS using CH₄/NH₃, (96:4) as reagent gas. The oleate hydroperoxides gave diagnostic fragmentation ions that occurred via scission α to the hydroperoxy group with loss of water (66). The following diagnostic fragment ions were obtained: 11-cis-OOH (m/z 213; 230 as ammonia adduct); 10-trans-OOH (m/z 199; 216 as ammonia adduct); 9-trans-OOH (m/z 155; 172 as ammonia adduct); and 8-cis-OOH (m/z 169; 186 as ammonia adduct). The first and second eluting HPLC fractions were identified as the 11-cis and 11-trans oleate hydroperoxides. The third peak contained both the 10-trans and 9-trans compounds, and the fifth and sixth elution fractions were identified as the 8-oleate hydroperoxides, with the 8-cis compound eluting before the 8-trans. The 9- and 10-trans compounds, however, coeluted on silica. In contrast, the alcohols that result from reduction of these hydroperoxides separate by normal phase HPLC, and a complete analysis of the products can be achieved using UV detection at 205 nm (66). On normal phase HPLC (two 5 µ silica columns, with 0.6% 2-propanol in hexane) the isomeric hydroxides emerge in the order of their polarity: 11-trans (16%); 11-cis (13%); 10-trans (21%); 9-trans (21%); 8-trans (16%); and 8-cis (13%). The proportions of the products, however, vary with the concentration of the starting oleate and it may also vary with the exact nature of the oleate ester. Furthermore, in concentrated solutions, hydroperoxides form dimers. Hydroperoxide loading, which greatly accelerated the reaction of the oxidation mixture yielded only the 11-cis, 10-trans and 8-trans hydroperoxides, which were resolved as the alcohols by normal phase HPLC as described (66).

The autoxidation of methyl linoleate has also been extensively studied (64,65,67), and the four major products of this reaction are the hydroperoxides: 13-trans,cis; 13-trans,trans; 9-trans,cis and 9-trans,trans (67). Autoxidation was carried out in air and initiated with di-tert-butyl peroxyxalate. The methyl linoleate hydroperoxides are extensively resolved by normal phase HPLC in order of increasing polarity: 13-hydroperoxy-cis, trans-11-octadecadienoate < methyl 13-hydroperoxy-trans, trans-9, trans-11-octadecadienoate < methyl 9-hydroperoxy-trans, cis-12-octadecadienoate < methyl 9-hydroperoxy-trans, trans-12-octadecadienoate (67). A complete resolution of the hydroperoxy diene products was obtained following reduction to the corresponding alcohols with NaBH₄ and analyzed by HPLC (Partisil-5 with 0.75%
ethanol n hexane). The alcohols were eluted in order of increasing polarity: methyl 13-hydroxy-cis-9, trans-11-octadecadienoate < methyl 13-hydroxy-trans-9,trans-11-octadecadienoate < methyl 9-hydroxy-trans,10,cis-12-octadecadienoate < methyl 9-hydroxy-trans-10,trans-12-octadecadienoate.

Plattner and Gardner (68) determined the CI spectra of intact isomeric 9- and 13-hydroperoxy linoleates using direct probe. With isobutene abundant fragment ions were observed due to the loss of H2O2 (m/z 295), H2O (m/z 309), and O (m/z 311). With ammonia, abundant ammonium adduct ions were observed (e. g. m/z 344 for methyl 9-hydroperoxy-trans-10,cis-12-octadecadienoate. The ammonium adduct ions were more stable than the [M+H]+ ion formed using methane or isobutene. The CID daughters of the high mass ions could be used to identify the position of the hydroperoxy group in the isomers studied.

Based on the understanding of methyl linoleate peroxidation as a free radical chain reaction, the three primary products of autoxidation were predicted to be the 9-, 11-, and 13-hydroperoxides. The 9- and 13-hydroperoxides were found readily, but formation of 11-hydroperoxides or any other bis-allylic fatty acid hydroperoxide had not been demonstrated until recently. Brash (69) has now shown that in vitamin E-controlled autoxidation of methyl linoleate, the 11-hydroperoxy derivative is the next most prominent primary peroxidation product after 9- and 13-hydroperoxides. The 11-hydroperoxide was not detectable in the absence of a-tocopherol. From a normal phase HPLC column the 11-hydroperoxide emerged immediately after the 13-hydroperoxy-trans,trans isomer. The structures of 11-hydroperoxylinoleate and its 11-hydroxy derivative were established by HPLC, GC-MS and NMR. When analyzed as the methyl ester TMS ether of the triphenylphosphine-reduced (hydroxy) derivative, the novel product had a mass spectrum similar to 9- and 13-hydroxylinoleates. The most prominent ions were present at m/z 382 (M, 35% relative abundance), m/z 311 (M-C7H5, 48%), m/z 225 (C9H7), base peak), and m/z 130 (55% relative abundance). The position of the hydroxyl group in the new product was established unambiguously from the mass spectrum of the methyl ester TMS ether derivative of the hydrogenated product. The two most prominent ions above m/z 100 were the two α-cleavage ions at m/z 287 and 201 (26 and 62% relative abundance, respectively).

Methyl linoleate has two bis-allylic methylene groups and reacts twice as fast with oxygen as linoleate (70). Reaction with oxygen at the end-carbon positions of each pentadienyl radical produces a mixture of four positional peroxy radicals leading to the corresponding conjugated diene 9-, 12-, 13- and 16-hydroperoxides containing a third isolated cis-double bond: 9-hydroperoxy-trans-10, cis-12, cis-15-octadecatrienoate; 13-hydroper oxy-cis-9,trans-11,cis-15-octadecatrienoate; 12-hydroper oxy-cis-9,trans-13, cis-15-octadecatrienoate; 16-hydroper oxy-cis-9,cis-12,trans-14-octadecatrienoate. In contrast to methyl linoleate hydroperoxides, those of linoleate were not resolved by normal phase HPLC but gave one composite peak (70). However, following reduction to the hydroxylinolenates, the mixture was resolved into eight major geometric components. The mass spectra indicated that each component consisted essentially of one single positional isomer with a small degree of contamination from adjacent isomer(s). On basis of UV and infrared spectra and chemical transformation, the HPLC peaks (I-VIII) were assigned the following identity (in order of increasing polarity): (Peak 1) methyl 13-hydroxy-cis-9,trans-11,cis-15 octadecatrienoate; (Peak 2) methyl 12-hydroxy-cis-9,trans-13,cis-15 octadecatrienoate; (Peak 3) methyl 12-hydroxy-cis-9,trans-13,trans-15 octadecatrienoate; (Peak 4) methyl 13-hydroxy-trans,11,cis-15 octadecatrienoate; (Peak 5) methyl 16-hydroxy-cis-9,cis-12,trans-14 octadecatrienoate; (Peak 6) methyl 9-hydroxy-trans,10,cis-12,cis-15 octadecatrienoate; (Peak 7) methyl 16-hydroxy-cis-9,trans-12,trans-14 octadecatrienoate; (Peak 8) methyl 9-hydroxy-trans,10,trans-12,cis-15 octadecatrienoate. Characteristic fragment ions were obtained for the 9 (m/z 155), 12, (m/z 197), 13 (m/z 211), and 16 (m/z 253) isomers.

Oliw et al. (71) used normal phase HPLC with ion trap mass spectrometer to analyze the hydroperoxides of the main metabolites of oleic, linoleic, alpha-linolenic and gamma-linolenic acids, which are formed by manganese lipoxygenase and linoleate diole synthase. MS3 analysis of hydroperoxides and MS2 analysis of dihydroxy- and monohydroxy metabolites yielded many fragments with information on the position of oxygenated carbons. MLO oxygenated C13 and C15 of 18:2n6, 18:3n3, and 18:3n6 in a ratio of approximately 1:1:3 at high substrate concentrations. 8-Hydroxy-9(10)-epoxysteratae was identified as a novel metabolite of LDS and oleic acid.

Methyl arachidonate reacts with oxygen about twice as fast as linoleate because it has three active bis-allylic methylene groups and three 1,4-diene systems. By the same mechanism of autoxidation as methyl linoleate and linolenate, arachidonate produces three pentadienyl radicals. Oxygen attack at either end of these pentadienyl radicals produces a mixture of six positional isomers with hydroperoxide substitution on carbons 5, 8, 9, 11, 12 and 15 (65,72). Free radical oxidation of arachidonic acid yields six simple hydroperoxide products. These compounds all have trans-cis diene geometry. As with linolenate, the outer 5- and 15-hydroperoxides of arachidonate are formed at higher concentrations (52%) than the inner 8-, 9-, 11- and 12-hydroperoxides (10-16%). Porter et al (72) have reported an HPLC separation of the products isolated from air oxidation of arachidonic acid. The hydroperoxides were eluted in order of increasing polarity as follows: (Peak 1) cis-trans-12; (Peak 2) cis-trans-15; (Peak 3) cis-trans-11; (Peak 4) cis-trans-9; (Peak 5) cis-trans-8; (Peak 6) cis-trans-5. A closely similar separation was obtained for the triphenylphosphine reduction products of autoxidized methyl arachidonate using a similar normal phase HPLC system, except for additional resolution of geometric isomers. The hydroxides of the methyl arachidonates were eluted in order of increasing polarity as follows: (Peak 1) cis-trans-12 (m/z 301,215); (Peak 2) cis-trans-15 (m/z 3210).
The fragmentation pattern of 12-HETE gave the expected isomers; however, the intensity of product ions was lower due to prior losses of water from the hydroperoxides. MS/MS of 5-HETE, which are easily decomposed and therefore difficult to analyze, produced ions similar to those found for low and high energy FAB-tandem MS of positional isomers of HETEs.

Production of HETEs by photoperoxidation yielded racemic mixtures of R- and S-isomers of hydroxyicosatetraenoic acids (HETEs). The stereochemical analysis of each HETE required prior purification of the isolated positional isomers of HETEs by normal phase HPLC with n-heptane/2-propanol/TFA and n-heptane/2-propanol/TFA as mobile phases. The chiral phase HPLC was performed on a Chiralpak AD column. The peaks were detected by UV absorption at 235 nm. In contrast to autoxidation, which produced only six conjugated positional isomers of HpETEs, seven positional isomers of HpETEs were found. The two supplementary isomers were the non-conjugated products, 14-HpETE and 6-HpETE, which were recognized from radiolabeling. Production of HETEs by photoperoxidation yielded racemic mixtures of R- and S-isomers with the S-isomers emerging ahead of the R-isomers (Figure 1).

With increasing number of double bonds there is an increase in the complexity of the mixtures of hydroperoxides, which are easily decomposed and therefore difficult to analyze. The most important are the cis-5,8,11,14,17-eicosapentaenoic acid (EPA) and all cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) found in fish and marine oils and represent the omega-3 isomers, which are of much interest to human lipid metabolism. The hydroperoxides produced from EPA have been identified but not quantified. Thus, EPA has been shown to yield eight isomers: 5-5-, 8-, 9-, 11-, 12-, 14-, 15-, and 16-hydroperoxides, while DHA produced ten positional isomers: 4-, 7-, 8-, 10-, 11-, 13-, 14-, 16-, 17-, and 20-hydroperoxides (64). VanRollins et al. (39) identified the liver microsomal metabolites of 20:5n3 as 17(18)-, 14(15)-, 11(12)-, and 8(9)-dihydroxyeicosatetraenoic acids, 20-hydroxyeicosapentaenoic acid, and 19-hydroxyeicosapentaenoic acid. The hydroxy fatty acids containing conjugated dienes were resolved by reversed phase HPLC and by normal phase HPLC. VanRollins and Murphy (40) resolved the isomers of methyl hydroxydocosahexaenoate into ten peaks by reversed phase HPLC and by normal phase HPLC. VanRollins et al. (39) identified the liver microsomal metabolites of 20:5n3 as 17(18)-, 14(15)-, 11(12)-, and 8(9)-dihydroxyeicosatetraenoic acids, 20-hydroxyeicosapentaenoic acid, and 19-hydroxyeicosapentaenoic acid. The hydroxy fatty acids containing conjugated dienes were resolved by reversed phase HPLC and by normal phase HPLC.

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Peak 8 << Peak 9+10, using the above peak identification. There was an extensive overlap between components Peak 10 and Peak 9. The peaks were identified by GC/EI-MS using a glass column interfaced with a quadrupole mass spectrometer (Model 3200, Finnigan, Sunnyvale, CA). The compounds were analyzed, before and after hydrogenation, as the TMS ethers, which yielded characteristic masses for α cleavage of the chain on the methyl and carboxyl side of the fatty acid derivative.

Lyberg and Adlercreutz (74, 75) have recently reported an HPLC method for the analysis of mono- and polyhydroperoxyDHA. The HPLC analysis was performed on a LaChrom system (Hitachi High technologies America, Schaumburg, IL) and an L-7400 UV detector. The column was a Lichrosphere 100 RP-18 (Merck, Darmstadt, Germany). For LC/MS, the flow from the column was led into the QSTAR Q-TOF hybrid tandem MS (PE Sciex, Toronto, Canada) with a TurbolonSpray source set to negative ion mode. A total of eight isomeric monohydroperoxide peaks were eluted by HPLC over a range of 19-36 minutes during chromatography of a bulk sample of DHA incubated at 6 °C for 3 days. Characteristic fragments were created when cleavage took place one carbon atom away from the hydroperoxide carbon. The fragment ions containing the functional group lost H2O molecule and were transformed into hydroxide, and a double bond was formed between the cleavage carbon and the carbon of the functional group. A determination of the characteristic fragment ions in the different isomers of monohydroperoxy DHA in the eight peaks of the chromatogram allowed the location of the hydroperoxide carbon (atom) to be determined. The hydroperoxides were found on C20 (Peak 1), C17 (Peak 2), C16 (Peak 2), C14 (Peak 3), C13 (Peak 3), C10 (Peak 4), C11 (Peak 5), C8 (Peak 7), C7 (Peak 8) and C6 (peak 8).

4.1.2. Secondary products

Prolonged oxidation of olate, linoleate and polyunsaturated fatty acids produce a multitude of secondary oxidation products. Methyl olate yields small amounts of allylic ketoleates (with CO on carbons 8-, 9-, 10-, and 11), epoxyoleate or epoxyoleates (8,9-, 9,10- and 10,11-epoxy), dihydroxyleoleates (8,9-, 9,10- and 10,11-diOH) and dihydroxysearates (between carbon 9 and carbon 11). It is believed (64,65) that the allylic ketoleates arise from dehydration of the corresponding hydroperoxides. The 9,10-epoxysearate may be produced by the reaction of olate and the hydroperoxides. The epoxyproducts may arise from cyclization of an alkoxy radical formed from the corresponding hydroperoxides of olate. The 1,2- and 1,4-dihydroxy esters may be formed from a similar alkoxy radical that undergoes hydroxyl and hydrogen radical substitution via an allylic hydroxy ester radical. Further oxidation of linoleate hydroperoxide yields ketoleinate (with CO on C9 and C13), epoxyhydroxyleoleate (9,10,12,13-epoxy-11-hydroxy and 9,13-hydroxy-12,13-9,10-epoxy), 9,13-dihydroxy, and trihydroxy (9,1,13- and 9,12,13-) stearate. Other polyxoygenated compounds formed by thermal decomposition of linoleate hydroperoxides include di- and trihydroxy esters. Linolenate hydroperoxidation yields epoxides, which decompose to cyclic peroxides, that undergo a second cyclization to produce bicyclo-endoperoxides and malondialdehyde.

With autoxidized olate, a mixture of 8-, 9-, 10- and 11-oxo esters is produced by cleavage A on the ester side of the hydroperoxides. With the monohydroperoxides of TAGs containing olate, the corresponding oxo acyglycerols are formed; these high-molecular weight non-volatile aldehydoacylglycerols, referred also as core aldehydes, are useful predictors of quality of edible oils (see below). Other cleavages of the hydroperoxides of olate result in volatile aldehydes, which are lost from the peroxidation residues of natural oils and fats and steryl esters. With autoxidized linoleate, 2,4-decadienal and methyl octanoate are produced by homolytic cleavage A on the 9-hydroperoxide, and 3-nonenal and 9-oxononanoate by cleavage B; hexanal, pentane, 1-pentanol and pentanal are produced from the 13-hydroperoxide (9).

Likewise, similar core aldehydes are generated by metal catalyzed homolytic cleavage of the hydroperoxides of linolenate, arachidonate and docosahexaenoate. Of interest to the LC-MS analysis of peroxidized TAGs and cholesterol esters are the homolytic cleavage products with the hydroperoxy group located on the carboxyl end of the molecule. Like the olete and linoleate, the monohydroxyperoxide of linolenate yields [9-oxo]nonanooate, while the arachidonate would yield the [5-oxo]valerate, but the docosahexaenoate, the [4-oxo]butyrate, which would e retained by their respective parent ester molecules (see below). At elevated temperatures, saturated aldehydes and dialdehydes are further oxidized into mono- and dibasic acids. The popular 4-hydroxy-nonenal is produced during homolytic decomposition of αβ polyunsaturated fatty acid hydroperoxides, via an 4-hydroperoxy-2-nonenal (3).

A large number of compounds have been identified from autoxidized methyl linoleate and further oxidation of linoleate hydroperoxides, including ketoleinate (with CO on C9 and C13), epoxyhydroxyleoleate (9,10,12,13-epoxy-11-hydroxy and 9,13-hydroxy-12,13-9,10-epoxy), 9,13-dihydroxy, and trihydroxy (9,1,13- and 9,12,13-) stearate. The epox allylic hydroxy, hydroperoxy and keto products can be formed by cyclization of an alkoxy radical formed from the corresponding hydroperoxides of linoleate (64). In contrast to thermal and metal catalyzed decomposition of hydroperoxides, which proceeds by homolytic cleavage, decomposition under acid conditions proceeds by a different mechanism involving heterolytic cleavage producing ionic ether intermediates, also referred to as carbocations (64,65).

Schneider et al. (37) utilized reversed phase HPLC for the analysis of the autoxidation products 13S-hydroperoxy-9Z,11E-octadecadienoic (13S-HPODE) and 9S-hydroperoxy-10E,12Z-octadecadienoic (9S-HPODE) acids. The column effluent was monitored with HP 1040 diode array detector. The [10R-3H]- and [10S-3H]arachidonic acids were used to demonstrate that prostaglandin ring formation by cyclooxygenase does not
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involve carbocation at C-10 of arachidonic acid as has been hypothesized previously.

Kim and Sawazaki (76) reported a structural analysis of the hydroxyl derivatives of docosahexaenoic acid as the PFB derivatives in the negative ion mode under filament – or discharge-on conditions, which generated abundant [M-PFB] ions. These ions were further fragmented by CID with argon and detected in the negative ion mode. Characteristic fragmentation occurred at the oxygenated C-C bonds, allowing unambiguous determination of the sites of oxygenation. Using this method the structures of epoxy, hydroxyl derivatives of 4,7,10,13,16,19-docosahexaenoic acid (22:6n3) formed by soybean lipoygenase were determined. They were identified as 13-hydroxy-16,17-epoxy-22:5n3 and 15-hydroxy-16,17-epoxy-22:5n3.

More recently, Bylund et al. (77) used reversed phase LC-MS with an ion trap mass spectrometer to resolve the metabolites of arachidonic and linoleic acids, including the epoxides, produced by human recombinant cytochrome P450 enzymes. The CYP2c9 converted arachidonic acid, octadecanetrahydroxyl acid, and linoleic acids to epoxides. Likewise, MacPherson et al. (78) reported the use of HPLC with ESI-MS to identify the arachidonic acid metabolites of Limulus amoebocytes.

Pruzanski et al. (79) employed LC/ESI-MS in the negative ion mode to identify the peroxy fatty acids released by group IIa secretory phospholipase A2 from the autoxidized PtdCho of HDL and acute phase HDL. Mono- and dihydroperoxy linoleic and arachidonic acids were recognized as early emerging peaks from a normal phase HPLC column eluted with a gradient of CHCl3/MeOH/NH4OH.

Pfister et al. (80) used chemical derivatization and reversed phase HPLC along with GC-MS to identify 11,14,15- and 11,12,15-trihydroxyeicosatetraenoic acids in rabbit aortic endothelial cells, where they act as relaxing factors of aorta. For EI or positive ion CI-MS, the sample was dissolved in 120 µl of CH3CN, esterified with diazomethane, and trimethylsilylated. For negative CI-MS, PFB esters and TMS ethers were prepared. The analyses were performed on a Hewlett-Packard 5890 series gas chromatograph equipped with a 14 m capillary DB-5 column, coupled to 5989A mass spectrometer. It was suggested that arachidonic acid is metabolized by 15-lipoxygenase to 15-HpETE, which undergoes an enzymatic rearrangement to 11-epoxy-14,15-EETA and 15-epoxy-11,12-EETA. Hydrolysis of the epoxy groups results in the formation of 11,14,15- and 11,12,15-trihydroxyeicosatetraenoic acids.

Kanazawa and Ashida (81) reported the identification of the epoxyketones, 11-oxo-12,13-epoxy-9-octadecenoic acid and 11-oxo-9,10-epoxy-12-octadecenoic acid as degradation products of linoleic acid hydroperoxides in the gastrointestinal of rats. The new compounds were characterized by reversed phase HPLC and GC/MS and the identification confirmed by infrared and laser Raman spectrometry. The hydroperoxides of linoleic acid decomposed in the stomach to 9-oxo-nonanoic acid and hexanal from beta-scission of 9- and 13-hydroperoxylinoelcic acid (82). The aldehydes were determined as the DNPBs by reversed phase HPLC using a linear gradient of methanol in water and monitoring at 360 nm. The peaks were identified by comparison of retention time of standards.

Santiago-Vazquez et al. (83) have described the use of LC-APCI-MS for the analysis of algal hydroxy fatty acid methyl esters. In an organic solvent extract of a cell free incubation mixture of Euglena gracilis, four peaks were discerned with retention times similar to those of commercial standards with m/z 317, which corresponds to the major ion in many of the arachidonic acid derived hydroxy fatty acids. The peaks were identified as the methyl esters of 15-HETE ME, 12-HETE ME, 8-HETE ME and 5-HETE ME. A detection limit of 20 picogram/cmicroliter per injection was obtained for 5-HETE ME based on signal to noise ratio of the m/z 317 ion, which corresponds to the loss of a hydroxyl group [M-17].

The peroxyl radicals of linolenate, arachidonate and docosahexaenoate yield cyclic peroxides (64), which can undergo a second cyclization to produce bicyclo-endoperoxides. Frankel et al (84) used CI-MS with a direct exposure probe to analyze a series of hydroperoxy cyclic peroxides and dihydroperoxides obtained from methyl linoleate and linolenate. Figure 2 shows the isobutene (A) and ammonia (B) CI-MS of methyl 9-hydroperoxy-10,12-epidioxy-13-octadecenoate. The mass spectra obtained with isotobane and ammonia as reacting gases showed fragmentation patterns similar to those previously observed by thermal decomposition under GC/EI-MS conditions. Abundant ions at m/z 113 resulted from the direct cleavage of the cyclic peroxide structure derived from linoleic acid as well as an ion at m/z 187 corresponding to cleavage between the hydroperoxy and cyclic peroxide with further loss of an oxygen atom.

The hydroperoxides of arachidonic and docosahexaenoic acids yield isoprostanes and neuroprostanoids, which initially are formed as oxoglycerophospholipid derivatives, but may also be detected as oxolipid moieties in cholesteryl esters and TAGs (see below). Utilizing mass spectrometric analyses, Bernoud-Hubac et al. (44) found that neuroketsals were formed in abundance in vitro during oxidation of docosahexaenoic acid. The neuroketsals were shown to rapidly adduct to lysine, forming lactam and Schiff base adducts. Furthermore, neuroketal, lysyl-lactam protein adducts were detected in nonoxidized rat brain synaptosomes at a level of 0.09 nanogram/mg protein, which increased 19-fold following oxidation in vitro. The predicted [NH4]+ ion for the dehydrated reduced Schiff base NK lysine adduct is m/z 491. The predicted NK lysine lactam adduct is m/z 503. The presence of multiple m/z 491 and m/z 503 peaks was consistent with the formation of multiple NK-lysyl adduct isomers.
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Figure 2. Isobutane CI-MS (A) of methyl-9-hydroperoxy-10,12-epidioxy-13-octadecanoate and ammonia CI-MS (B) of methyl 9-hydroperoxy-10,12-epidioxy-13-octadecanoate. Abundant ions at \textit{m/z} 113 resulted from direct cleavage of the cyclic peroxide derived from linoleic acid methyl ester, while an ion at \textit{m/z} 187 resulted from a cleavage between the hydroperoxy and cyclic peroxide functions with a further loss of an oxygen atom. Finnigan 4535/TSQ and CI-MS with direct exposure probe was used as previously described by Plattner and Gardner (68). Reproduced with permission from (84).

The decomposition of the hydroperoxides of the polyunsaturated fatty acids yield the stable C$_5$ and C$_4$-aldehydes, while the oligoenoic fatty acids yield the C$_9$-aldehyde as major components (9).

4.2. Oxoradylglycerols

The oxoradylglycerols encompass both acyl and alkyl derivatives of glycerol.

The alkyl ethers are usually C$_{16}$ and C$_{18}$ saturated, but monounsaturated species are also found. The glyceryl esters contain mainly oleic, linoleic, arachidonic and docosahexaenoic acids, which upon autoxidation yield primary and secondary autoxidation products similar to those produced from the corresponding fatty acid methyl esters, except that more than one hydroperoxy fatty acid may occur per acylglycerol molecule.

4.2.1. Primary oxidation products

The hydroperoxides of monoradylglycerols arise from autoxidation of unsaturated monoalkylglycerol ethers or unsaturated monoacylglycerols. In addition to the geometric and enantiomeric isomers of the hydroperoxides, the oxomoaoyl-glycerols exhibit both positional and enantiomeric isomerism of the acylglycerol molecule. Sn-1-monoalkylglycerol hydroperoxides result from phospholipase hydrolysis of alkylglycerophospholipids, while sn-1-monoacylglycerol hydroperoxides arise from phospholipase C hydrolysis of the hydroperoxides of lysoglycerophospholipids. Sn-2-monoacylglycerols result from pancreatic lipase hydrolysis of TAG hydroperoxides. The monoradylglycerols may also be converted into hydroperoxides by direct autoxidation.

Kozak et al. (85) demonstrated that soybean 15-LOX, rabbit reticulocyte 15-LOX, human 15-LOX-1 and human 15-LOX-2 oxygenated sn-2-monoarachidonylglycerol (AG) to yield 15(S)-hydroperoxyeicosatetraenoic acid glyceryl ester. Steady state kinetics demonstrated that 15-LOX-1 and 15-LOX-2 oxygenate 2-AG comparably or preferably to arachidonic acid. The product of 2-AG metabolism by soybean 15-LOX was established by chromatography, UV spectroscopy, and MS/MS. Direct infusion of the organic extract of 2-AG/soybean 15-LOX incubations into the mass spectrometer revealed a single predominant product with a mass to charge ratio of 433 consistent with a HpETE-G sodium adduct (Figure 3). CID of this metabolite produced the expected hydroperoxide cleavage and established the C-15 regiochemistry of 2-AG oxygenation by 15-LOX. In addition to the major ion at \textit{m/z} 433, minor ions were detected with mass to charge ratios of 499 and 465 corresponding to the sodium adduct of HpETE-G species and the sodium adduct of a bis-dioxygenation product (M+2O$_2$+Na$^+$). The product of 2-AG metabolism by rabbit 15-LOX was shown to give a similar product. In contrast to 15-LOX-1, which, in addition to the C$_{15}$ oxygenated product gave also a small amount of the C$_{12}$ product, 15-LOX-2 catalyzed C-15 hydroperoxidation regiospecifically; no significant 12-HETE was detected following reduction and saponification.

Hydroperoxides of sn-1,2-diradylglycerols are released by phospholipase C hydrolysis of autoxidized glycerophospholipids (25), while pancreatic lipase releases a mixture of hydroperoxide-containing 1,2(2,3)-DAGs and 2-MAGs from autoxidized TAGs (86). Like the hydroperoxides of monoradylglycerols, the hydroperoxy DAGs possess in addition to the positional and geometric isomers, also positional and chiral isomers of the DAG parent molecule. While the positional isomers are resolved by normal phase HPLC, the enantiomers require chiral phase HPLC (8). Simple separations and identification based on molecular weight may be obtained by GC-MS of the TMS or tert-BDMS ethers (25).

It has been estimated that soybean oil containing a mixture of oleate, linoleate and linolenate TAGs would yield 14 positional hydroperoxides by autoxidation. Neff et al. (86) reported the separation of autoxidation products of trilinoleoylglycerols (LLL) by reversed phase and normal phase HPLC. The autoxidation products were characterized by GLC and GC-MS of the component fatty acids. Preparative reversed phase HPLC of autoxidized
Oxolipids

Figure 3. Mass spectrometry of oxygenated sn-2-arachidonoylglycerol products. Structure of the 15(S) hydroperoxyeicosatetraenoic acid glycerol ester as obtained by oxidation with 15-LOX of soybean. (A) representative direct infusion, positive ion, ESI-MS of sn-2-arachidonoylglycerol metabolites produced by treating 10 microg of endocannabinoid with 10 microg of soybean 15-LOX (37 °C, 10 min) in 100 microl. of 25 mM Tris, 0.015% Tween 20, pH 7.4; (B) CID spectrum of primary oxidation products. The purified sample was redissolved in MeCN/H2O (1:1, v/v) and infused into the mass spectrometer. Direct infusion MS was conducted with solutions of 10-100 microg/ml in MeOH/H2O or MeCN. CID was accomplished with argon as the collision gas. The instrument was a Finnigan TSQ-7000 triple quadrupole MS. Regiochemistry was established by diagnostic, CI hydroperoxide cleavage (85,95). Reproduced with permission from (95).

LLL yielded mono- (28.4%), bis- (10.9%) and tris- (1.7%) hydroperoxides. Subsequent analytical normal phase HPLC of the monohydroperoxide mixture yielded all eight positional and geometric monohydroperoxide isomers expected from LLL. In order of increasing retention time, they were sn-2-[cis,trans-13-hydroperoxy]- < l(3)-[cis,trans-13-hydroperoxy]- < sn-2-[trans,trans-13-hydroperoxy]- < l(3)-[trans,trans-13-hydroperoxy]; sn-2-[trans,trans-9-hydroperoxy]- < l(3)-[trans,trans-9-hydroperoxy]- < l(3)-[cis,trans-9-hydroperoxy]- < sn-2-[cis,trans-9-hydroperoxy]- glycerol-trilinolenates. The main primary products included mono-, bis- and tris-9-hydroperoxy-trans-10,12-; 9-hydroperoxy-trans-10,trans-12; 13-hydroperoxy-cis-9,trans-11-; and 13-hydroperoxy-trans-9,trans-11-linoleoylglycerols.

Francel et al. (87) used HPLC to purify the hydroperoxides resulting from autoxidation of trilinolenoylglycerol (LnLnLn) at 40 °C and identified either as intact TAGs spectrophotometrically, or after lipolysis as the fatty acid TMS derivatives by capillary GLC and GC-MS. The products included 9-, 12-, 13-, and 16-mono-, bis-, and tris-hydroperoxides as primary products of autoxidation. The bis- and tris-hydroperoxides were formed consecutively as minor products from the monohydroperoxides. Analytical normal phase HPLC of the monohydroperoxide mixture from LnLnLn yielded the following elution order: sn-2-[cis,trans-12(13)-hydroperoxy]- < sn-2-[cis,cis,trans-16-hydroperoxy]- < l(3)-[cis,trans,cis-12(13)-hydroperoxy]- < 1(3)-[cis,cis,trans-16-hydroperoxy]; sn-2-[trans,cis,cis-9-hydroperoxy]- < l(3)-[trans,cis,9-hydroperoxy]-glycerol trilinolenate. The triacylglycerol position of the monohydroperoxides of LnLnLn was determined by analytical normal phase HPLC. Six peaks were resolved and identified according to the same scheme used for the monohydroperoxides of LnLnLn, by pancreatic lipase hydrolysis and capillary GLC after hydrogenation and methylation. The monohydroperoxides were eluted in order of increasing polarity as follows: Peak 1, 2-mono-cis,trans,cis-12(13)-hydroperoxide; Peak 2, 2-mono-cis,cis,trans-16-hydroperoxide; Peak 3, l(3)-mono-cis,cis,trans-12(13)-hydroperoxide; Peak 4, l(3)-mono-cis,cis,trans-16-hydroperoxide; Peak 5, 2-mono-trans,cis,9-hydroperoxide; Peak 6, l(3)-mono-trans,cis,9-hydroperoxide.

Kuksis et al. (88) used reversed phase HPLC along with on-line TSI-MS and ESI-MS to identify the mixed hydroperoxides of synthetic and natural TAGs following reaction with tert-butyl hydroperoxide/Fe2+ reagent. Both primary and secondary oxidation products were recovered together. The hydroperoxides were identified on the basis of previous chromatographic work of Neff et al. (86) and Frankel et al. (87) and by on-line TSI-MS, which confirmed the masses of the hydroperoxides and hydroxides.

Sjovall et al. (47) later reported the reversed phase LC/ESI-MS separation and identification of the bis and mono-hydroperoxides of 1,2-dilinoleoyl-3-stearoylglycerols along with a partial resolution of regioisomers. In the positive ion mode, mass values corresponding to the [M+Na]+ adducts at m/z 969 and m/z 937, respectively, were obtained for the bis and mono-derivatives. The HPLC resolution was effected with a Supelcosil LC-18 column and a linear gradient of 2-
propanol in MeOH, while a single quadrupole mass spectrometer (Hewlett-Packard Model 5989A) provided the mass spectra.

Miyazawa et al. (32) used reversed phase HPLC (C18 column, MeOH or MeOH:EtOH (5:1, v/v)) and FAB-MS (JOEL JNM-HX-105, JOELCO, Tokyo, Japan) to analyze the photoperoxidation products of trioleoylglycerol (OOO). Preparative HPLC with chemiluminescence allowed the detection and collection of three peaks corresponding to \( \text{tris-} \), \( \text{bis-} \) and mono-hydroperoxides in order of elution. Following reduction and hydrogenation, Peak 1 showed a molecular ion \([M+1]^+\) at \( m/z \) 908 corresponding to tristearoylglycerol (SSS) mono-OOH, thus indicating that the Peak 1 compound was OOO mono-OOH. Similarly the compounds of Peaks 2 and 3 gave \([M+1]^+\) at \( m/z \) 924 and \( m/z \) 941, corresponding to \( \text{bis-} \) and \( \text{tris-} \) hydroxy SSS after reduction and hydrogenation, thus indicating that Peak 2 and Peak 3 compounds were \( \text{bis-} \) OOHH and \( \text{tris-} \) OOHH, respectively, of OOO.

Kusaka et al. (89) reported compositional analysis of normal plant TAGs (corn, olive and perilla) and hydroperoxidized \( \text{rac-1-} \) stearyl-2-oleoyl-3-linoleoyl-sn-glycerols by HPLC in combination with APCI/MS (LC/APCI-MS). TAGs irradiated with a tungsten lamp (40 W) gave characteristic fragment ions \([M-H_2O+H]^+\), \([M-H_2O_2+H]^+\) and \([M-R_1(R_3)COOH+H_2O+H]^+\), which could be used to discriminate between fatty acids in \( sn-1 \) (or \( sn-3 \)) and \( sn-2 \) positions. The analyses were performed on a Hitachi (Tokyo, Japan) M-2000 double-focusing mass spectrometer equipped with a Hitachi L-6200 HPLC instrument and a Hitachi APCI interface system. Reversed phase LC/APCI-MS of the plant TAGs gave mass spectra with \([M+H]^+\) and \([M-R_1(R_3)COOH+H]^+\) ions, where the protonated molecular ion and fatty acid at the \( sn-1 \) (or \( sn-3 \)) position of the TAG. It was possible to discriminate between fatty acids in \( sn-1 \) (or \( sn-3 \)) and \( sn-2 \) positions. LC/APCI-MS of hydroperoxidized TAGs gave characteristic fragment ions \([M-H_2O_2+H]^+\), \([M-H_2O+H]^+\) and \([M-R_1(R_3)COOH+H_2O+H]^+\).

Neff and Byrdwell (90) coupled the reversed phase HPLC columns directly to a mass spectrometer via an APCI source to identify the intact TAG oxidation products as they eluted. In all cases (OOO, LLL, and LnLnLn) the primary oxidation products were TAGs containing mono-OOH functional groups, although many other products were also present in smaller amounts. The dramatic differences in the relative proportions of fragments arising from APCI-MS of molecules of different classes having identical masses highlighted the utility of this soft ionization technique. Only a small amount of protonated molecule was observed, with the primary high mass fragments being produced by sequential loss of portions of the hydroperoxide group. The first primary fragment formed was loss of the outer \( \text{–OH} \) from the hydroperoxide group followed by cyclization of the remaining oxygen to form an epoxide, resulting in loss of another hydrogen at the site of cyclization, for a net loss of 18 amu. The second primary high mass fragment was formed by complete loss of the hydroperoxy group along with a neighboring hydrogen to form an additional site of unsaturation. Also observed for all hydroperoxides was formation of an ion having even one less site of unsaturation. Only very small abundances of DAG fragment ions containing intact hydroperoxy groups were observed. The fragmentation of OOO mono-OOH to form the epoxide and loss of a specific hydrocarbon length allowed identification of the position of the epoxide ring. Across all oxidation products, the most important adducts, which were formed, were \([M+18]^+, [M+23]^+\) and \([M+39]^+\) adducts. Surprisingly, these adducts were attributed to CH3CN in the column effluent, although they would appear to correspond to adducts of ammonia, sodium and potassium, respectively. Another set of major oxidation products which was produced by these three standard TAGs were the \( \text{bis-} \) hydroperoxides. The fragmentation pathways were the same as those for the mono-hydroperoxides, except that two hydroperoxide groups were available to exhibit such fragmentation.

Kanazawa and Ashida (81, 82) resolved the mono-OOH of LLLs resulting from photosensitized oxidation with methylene blue (48) of LLL by reversed phase HPLC into trihydroperoxides (5.4 min), \( \text{bis-} \) hydroperoxides (5.9 min), \( \text{bis-} \) hydroperoxides (7.2 min), \( \text{bis-} \) hydroperoxides (8.2 min), \( \text{mono-} \) hydroperoxides (11.3 min), \( \text{mono-} \) hydroperoxides (14.8 min), and \( \text{mono-} \) hydroperoxides (19.5 min). These peaks were confirmed to be hydroperoxides by reduction with NaBH4. The isomeric composition of the linoleic acid hydroperoxides was determined by normal phase HPLC with hexane/diethyl ether, which eluted linoleic acid at 2.1 min, and the four isomers of linoleic acid hydroperoxides at 5.4 min (13-(Z,E)-hydroperoxide), 5.8 min (13-(E,E)-), 6.2 min (9-(E,Z)-), and 6.5 min (9-(E,E)-) and four isomers of the linoleic hydroxide at 11.5 min (13-(Z,E)-hydroxide), 14.3 min (13-(E,E)-), 16.6 min (9-(E,Z)-), and 19.5 min (9-(E,E)-). These isomers were identified by comparing retention times to those of the products of soybean lipoxygenases.

Bauer-Plank et al. (91) reported analysis of TAG hydroperoxides in vegetable oils by non-aqueous reversed phase HPLC with UV detection. The \( \text{mono-} \) and \( \text{bis-} \) hydroperoxides of LLL, LLO, and LOD were identified. Excellent separations were obtained for positional isomers of TAG hydroperoxides as demonstrated by the separation of 1-palmitoyl-2-(13-hydroperoxylinoleoyl)-3-stearoyl and 1-palmitoyl-2-stearoyl-3-(13-hydroperoxylinoleoyl)glycerol using a gradient of CH3CN in methylbutyl ether.

Sjovall et al. (92) reported the reversed phase LC/ESI-MS separation and identification of the TBHP oxidation products of unsaturated TAGs. Synthetic TAGs containing one (18:0/18:1/18:0 and 18:1/16:0/16:0) and two (18:0/18:0/18:2 and 18:1/18:1/18:0) double bonds per molecule were exposed to TBHP in presence of Fe(SO)4 and taurocholic acid and the reaction products resolved by HPLC using a linear 20-80% gradient of 2-propanol in MeOH. The resolved components were identified by online ESI-MS and LC/CID/ESI-MS. Hydroperoxides,
Hydroperoxide TAGs were formed from PPO and POP under both air and \(^{18}\)O atmosphere. The extracted spectrum of the peak supposed to be 1,2-dipalmitoyl-3-hydroperoxoyleoylglycerol, shows an intense ammonium adduct at \(m/z \) 883.0 \([M(NH_4)^+]\) (Figure 4). Fragmentation of the precursor at \(m/z \) 883.0 shows major product ions at \(m/z \) 866.0, 609.5, 591.8, 575.7 and 551.8 attributed to \([M+H]^+\), \([M-P]^+\), \([M-P-H_2O]^+\), \([M-P-H_2O_2]^+\) and \([M-HpoO]^+\), respectively. The product ion at \(m/z \) 609.5 was formed from the loss of palmitoyl chain (256.4 amu) from the protonated molecule at \(m/z \) 866.0. The neutral loss of hydrogen peroxide is specific to the hydroperoxide moiety and can be used for structural identification of hydroperoxyyl group containing molecules. The product ion at \(m/z \) 551.8 \([M-HpoO]^+\) was formed by the loss of the hydroperoxoyleoyl chain from the protonated molecule. Analyses of hydroperoxy-TAGs products formed from POP gave rise to similar observations. The 1,3-dipalmitoyl-2-hydroperoxyoleoyl (PPHpoO) fragmented with \([M-(^{18}\text{O})\text{HpoO}]^+\) DAG had a product intensity higher than \([M-P-H_2O]^+\) DAG, while PPHpoOP with \([M-(^{18}\text{O})\text{HpoO}]^+\) DAG had a product intensity lower than \([M-P-H_2O]^+\) DAG. Thus, both PPHpoO and PPHpoOP isomers had distinct DAG ratios in their fragmented spectra that could be used to distinguish and quantify regio-isomers (94).

### 4.2.2. Secondary oxidation products

Thus, 1(3) and 2-monoacylglycerols containing epoxy and hydroxy and aldehydic fatty acids have been recognized among the lipolysis products of autoxidized TAGs. Kozak et al. (85) used LC/ESI-MS/MS to demonstrate that 2-arachidonoylglycerol (2-AG) is a substrate for cyclooxygenase 2 (COX-2), which provides the novel lipid, prostaglandin H\(_2\) glycerol ester (PGH\(_2\)-G) \textit{in vitro} and in cultured macrophages. Reversed-phase LC/MS was conducted with a Waters 2690 Separations Module with a Zorbax RX-C\(_{18}\) narrow bore column (15 cm x 2.1 mm, 5 microm) interfaced to a Finnegan TSQ-7000 triple quadrupole mass spectrometer. Sodiated analytes were eluted with increasing concentrations of MeCN in 0.001% aqueous NaOAc. Selected ion monitoring and quantification was accomplished using pentadeuterated glyceryl prostaglandin standards. CID was accomplished with Ar as the collision gas. Subsequent work (95) has shown that bovine prostacyclin synthase catalyzes the isomerization of the intermediate endoperoxide, PGH\(_2\)-G to the corresponding prostacyclin derivatives. Figure 5 shows representative direct infusion, positive ion, ESI-MS profiles of 2-arachidonoylglycerol metabolites produced by treating 15 microg of 2-AG with (A) 1 microg of purified human COX-2 for 2 min at 37 °C or (B) with 30 microg of purified human COX-2 for 2 min at 37 °C followed by reduction with 15% Na\(_2\)S\(_2\)O\(_4\) for 20 min at room temperature (45). Direct infusion MS showed two major product masses, one at \(m/z \) 449 and the other at \(m/z \) 417, which corresponded to the sodiated molecular ions of the glyceryl esters of PGH\(_2\), PGE\(_2\), or PGD\(_2\), and 11- or 15-HETE, respectively. LC/MS revealed the presence of four primary products in 2-AG/COX-2 reaction mixtures. Two closely eluting polar metabolites each displayed \(m/z \) 449 consistent with PGE\(_2\)-G and PGD\(_2\)-G. Two closely eluting nonpolar 2-AG metabolites were detected with an \(m/z \) 417 consistent with...
Figure 5. Mass spectrometry of oxygenated sn-2-
arachidonoylglycerol products. Representative direct liquid infusion, positive ion, ESI-MS profile of sn-2-
arachidonoylglycerol metabolites produced by treating 15 microg 2-AG with (A) 15 microg of purified human CO-2 for 2 min at 37 °C or (B) 30 microg of purified human COX-2 for 2 min at 37 °C followed by reduction with 15% Na2S2O4 for 20 min at room temperature. Direct infusion MS was generally conducted with MeCN/H2O or MeOH/H2O. CID was accomplished with argon as the collision gas. Chemical structures indicate the assignment for the most abundant product with the appropriate mass to charge ratio. The instrument was a Finnigan TSQ-7000 triple quadrupole mass spectrometer. Reproduced with permission from (45).

glycerol esters of HETEs. Products were eluted with a 5 min gradient of 10-40% CH3CN in H2O (0.001% NaAc) followed by isocratic elution with 40% CH3CN. Similarly the identity of 6-keto-PGF1α was confirmed by selected ion mass chromatograms obtained for the unknown and the synthetic product of the EDCI-mediated coupling of 6-

Kurvinen et al. (96) used ozonization (50) to prepare core aldehydes of monoacylglycerol and characterized them by HPLC as the DNPH derivatives. The monoacylglycerol C8 core aldehyde reacted readily with aminophospholipids, amino acids and peptides to form Schiff bases. Figure 6 shows the reversed phase HPLC/ESI-MS of NaCNBH4-reduced Schiff base of 2-

Since non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed in situ on phospholipids (44), it would be anticipated that sn-1,2-DAGs containing isoprostane residues would be released by phospholipase C from peroxidized glycerophospholipids. Kamido et al. (25) reported the GLC separation of the 1,2-diacylglycerol core aldehyde moieties of egg yolk PtdCho as obtained by phospholipase C digestion, trimethylsilylation and its MOX derivative. In each case two peaks corresponding to the 1-

Tokumura et al. (97) identified sn-2-[omega-

Kurvinen et al. (96) used similar LC/ESI-MS methods to demonstrate the formation of the 2-MAD-ALD adducts of valine, lysine, as well as PtdEtn in addition to the three tripeptides.
Figure 6. Reversed phase LC/ESI-MS of NaCNBH₃-reduced Schiff base of 2-MG-ALD and glycine-histidine-lysine (GHK) as total negative ion current (A) and total mass spectrum of Schiff base peak (B), where deprotonated molecular ions for the reduced Schiff base of GHK and 2-MAG-ALD (m/z 569) and unreacted peptide (m/z 339) were the only ions detected. The schematic structure represents the most probable structure for the reduced Schiff base. The Schiff base adduct of GHK eluted at the beginning of the run (2.1 min), but not with the solvent front. The adducts of GGH and GGG eluted at 2.0 and 2.2 min, respectively. LC/ESI-MS consisted of Hewlett-Packard 1090 liquid chromatograph connected to a Hewlett-Packard 5989A quadrupole mass spectrometer equipped with a nebulizer-assisted ESI interface. Reduced Schiff bases of 2-MAG-ALD and peptides were analyzed using a reversed phase column (ODS Hypersil, 5 microm, 100 x 2.1 ID Hewlett-Packard) with a flow rate of 0.5 ml/min. The mobile phase composition was changed from 0.5% NH₄OH/MeOH/hexane (12:88:0 by vol) to (0:88:12 by vol) in 17 min after holding the starting composition for 3 min. Reproduced with permission from (96).

presence of C₄ core aldehydes in the oxoacylglycerol products of autoxidized TAGs of Baltic herring oil.

Frankel et al (87) isolated the 9- and 16-hydroperoxy epidioxylinolenoylglycerols as secondary products of LnLnLn autoxidation. Analytical normal phase HPLC separation of the mono-hydroperoxy epidoxides of LnLnLn showed eight peaks, which were eluted in order of increasing polarity: Peak 7+8, 2-mono-cis,trans-9-hydroperoxy epidioxide; Peak 9+10, 1(3)-mono-cis,trans-hydroperoxy epidioxide; Peak 11, 2-mono-cis,trans-16-hydroperoxy epidioxide; Peak 12, 1(3)-mono-cis,trans-16-hydroperoxy epidioxide; Peak 13, 2-mono-trans,trans-9(16)-hydroperoxide epoxide; Peak 14, 1(3)-mono-trans,trans-9(16)-hydroperoxide epoxide. The peaks due to hydroperoxide epoxides were identified by the same scheme as the mono-hydroperoxides. 2-Glycerol isomers of mono-hydroperoxy epoxides eluted in peaks 7, 8, 11 and 13, produced methyl stearate and 2-mono-9,10,12-(13,15,16)-tri-OTMS stearylglycerol (as TMS ethers) in a 2:1 ratio. 1(3)-Glycerol isomers of monohydroperoxy epoxides eluted in peaks 9,10,12 and 14, produced methyl stearate, methyl 9,10,12, or 13,15,16-tri-OTMS stearates and monostearoylglycerol (TMS ethers) in a 1:1:1 ratio. The cis,trans configuration of the isomers was established by NMR.

The products included 9-, 12-, 13- and 16-mono-, bis-, tris-hydroperoxy, and 9- and 16-hydroperoxy bicycloendoperoxylinolenoylglycerols, which again were
Figure 7. Reversed phase LC/ESI-MS analysis of autoxidized and DNPH derivatized 1-O-alkyl-2-oxoacyl-sn-glycerols from shark liver oil. (A) Total negative ion current profile of 1-O-alkyl-2-(DNPH-oxo)acyl-sn-glycerols. (B-G) reconstructed single-ion mass chromatograms of m/z 577, 579, 605, 647, 675, and 677 [M-1] diagnostic ions of 1-O-hexadecenyl-2-(DNPH-4-oxo)butyroyl-sn-glycerol, 1-O-hexadec-1-yl-2-(DNPH-4-oxo)butyroyl-sn-glycerol, 1-O-octadecenyl-2-(DNPH-4-oxo)butyroyl-sn-glycerol, 1-O-octadecyl-2-(DNPH-7-oxo)heptanoyl-sn-glycerol, 1-O-octadec-1-yl-2-(DNPH-9-oxo)nonanoyl-sn-glycerol, and 1-O-octadecenyl-2-(DNPH-9-oxo)nonanoyl-sn-glycerol, respectively. HPLC was performed with a Hewlett-Packard Model 1090 liquid chromatograph using an HP ODS Hypersil Ci8 column (5 microm; 200 x 2.1 mm ID) and eluted isocratically with 100% Solvent A (MeOH/H2O/30% NH4OH, 88:12:0.5, by vol) for 3 min, followed by a linear gradient of 0 to 100% Solvent B (MeOH/hexane/30% NH4OH, 88:12:0.5, by vol) in 25 min. On-line ESI-MS was performed by admitting the entire HPLC column effluent into a Hewlett-Packard Model 5989 B quadrupole mass spectrometer equipped with a nebulizer assisted EI interface. Nitrogen was used as both nebulizing (60 psi) and drying gas (60 psi) 270 °C. Reproduced with permission (98).
identified by GC-MS of the TMS derivatives. The monohydroperoxides and hydroperoxy epoxidases were the only main products initially formed. HPLC analyses showed a small preference for the formation of 16-hydroperoxides on the 1(3)-position over the 2-position of LnLnLn. There was no selectivity for the formation of the 9-, 12- and 13-hydroperoxides and for the hydroperoxy epoxidases between the 1(3)- and 2-positions in LnLnLn.

The three TAG standards (OOO, LLL and LnLnLn) produced a variety of other oxygen-containing compounds (90), which were identified by LC/APCI-MS, among these were stable epoxides formed by at least two distinct processes resulting in two types of epoxides. The first process was formation of the epoxide across the double bond in the TAG molecule, while the second was formation of the epoxide next to a double bond. In case of OOO, the formation of an epoxide at the double bond resulted in a single sharp chromatographic peak. These two distinct mechanisms accounted for the fact that epoxides which replaced a site of unsaturation had a protonated molecular ion and adduct ions which were 2 amu larger than the epoxides formed next to double bonds, but all these epoxides led to fragment ions having identical masses.

Neff and Byrdwell (90) used APCI-MS to identify the epoxidases and hydroperoxyepoxidases from the peroxidation of LnLnLn, which were not observed for the other TAGs (OOO and LLL). The molecular weight, fragmentation pattern, and the chromatographic retention time allowed the identification this molecule as epoxide (m/z 904.7), which was formed across an existing double bond. Another oxidation product was the hydroperoxide epoxidase (m/z 936.9). As with the mono-hydroperoxides, the major primary fragment arising from the hydroperoxide functional group was an epoxide formed by loss of an OH group, followed by cyclization with loss of an acyl chain hydrogen for a net loss of 18 amu, or H2O. The DAG ions confirmed the presence of the two functional groups identified in this class of molecules. The peak at m/z 641.5 came from the epoxide, epoxide fragment due to loss of 18 amu from the hydroperoxy group. The peak at m/z 625.5 arose from the diepoxide formed by loss of 18 amu from the hydroperoxy group and 16 amu from the epoxide group.

Giuffrida et al. (93) described the preparation and isolation of hydroperoxy and epoxy-TAGs containing oleic acid. The use of 18O2 oxidation experiments permitted unambiguous identification of the structures by MS/MS fragmentation. The reaction products were characterized by LC/ESI-MS/MS, FTIR and NMR analysis confirmed formation of epoxy moiety. The LC/ESI-MS/MS data were used to identify these compounds in a thermo-oxidized TAG mixture. The MS spectrum of PPEs (Figure 8) exhibits an intense ammonia adduct at m/z 867.0 [M(NH4)]. The fragmentation of the precursor at m/z 867.0 shows product ions at m/z 850.0, 593.8, 575.8 and 551.8 designated as [M−H], [M−P], [M−P−H2O] and [M−Es], respectively. Product ion at m/z 593.8, corresponding to a protonated epoxystearoyl DAG formed from the lost palmitoyl residue (P, 256.4 amu), from the protonated molecule at m/z 850.0. This DAG is prone to dehydration and gives a DAG ion at m/z 575.8. Such a dehydrated ion fragment is well characterized in the literature. The MS spectrum of synthetic PESP region-isomer shows a similar fragmentation pathway. The loss of the acyl chain from the external position of the precursor TAG ion is predominant (101). As a consequence, [M−P]/[M−Es] ratios (0.75 and 1.24) for PPEs And PESP, respectively) differ for individual regio-isomers and can be used to distinguish and quantify the two positional isomers as previously reported by Fauconnot et al. (94).

Earlier, Kuksis et al. (88) had used reversed phase HPLC with on-line TSI-MS to identify the epoxy and core aldehyde derivatives along with the hydroperoxides during oxidation of synthetic and natural TAGs with the tert-BOOH/Fe2+ reagent. The oxidation products were extracted with CHCl3/MeOH, reacted with DNPH, and resolved by normal phase TLC into fractions depending on the number of hydrazone derivatives formed. The individual TLC bands were examined by reversed phase HPLC. The HPLC separations were done on an HP-1090 liquid chromatograph equipped with a C18 Supelcosil column. A solution of 0.2 M NH4Ac/MeOH 1:1 (v/v) was added to the post column flow at a rate of 0.2 ml/min. The oxoTAGs were identified on basis of the single ion mass chromatograms for the major peroxidation products of 18:2/18:1/16:0 present in specific TLC bands. TAGs containing core aldehydes together with monohydroperoxides and epoxides were identified as the DNPH derivatives of the C9 aldehydes. The oxoTAG standards were resolved by normal-phase HPLC, and the identities of the oxoTAGs confirmed by LC/ESI-MS.

Later, Sjovall et al (92) used elution factors of oxoTAGs (47) as an aid in identification of secondary products of oxidation of reference TAGs by reversed phase HPLC. A total of 31 incremental elution factors were calculated from chromatography of 33 oxygenated and non-oxygenated TAG species, ranging in carbon number from 36 to 54 and in double bond number from 0 to 6. The chromatogram was complex and in contrast to the relatively simple full mass spectrum, which included both ammonia and sodium adducts. Single ion mass chromatograms were obtained for the major ions (as ammonia adducts) at m/z 882 (18:1/16:0/16:0, OOH+NH4), m/z 902 (18:0/16:0/16:0, OOH, epoxy+NH4), m/z 864 (18:1/16:0/16:0, epoxy+NH4), m/z 1026 (18:1/16:0/16:0, di-TBHP+NH4), m/z 938 (18:1/16:0/16:0, TBHP+NH4) and m/z 850 (18:1/16:0/16:0 +NH4). The tentative structures were proposed on basis of chromatographic retention time and mass spectra of reference compounds. Reduction of the oxidized 16:0/16:0/18:1 with NaN3 in CH3CN led to the conversion of the monohydroperoxides into the monohydroxides (m/z 866) and of the mono and di-tert-BOOH derivatives into the corresponding mono- and dihydroxide derivatives (m/z 866 and 882, respectively).

The major TAG from 2-4 h of peroxidation of 18:2/18:1/16:0 and corn oil TAG contained one unmodified FA in combination with 9-oxononoic acid and 9- or 13-
Figure 8. Mass spectra (A) of 1,2-dipalmitoyl-3-epoxystearoyl glycerol (PPEs) and (B) $^{18}$O-labelled 1,2-dipalmitoyl-3-epoxystearoyl-glycerol [PP($^{18}$O)HpoO]. Mass spectra were recorded on a Sciex API4000 triple quadrupole mass spectrometer (Applied Biosystems MDS Sciex, CA). The chromatographic separation was performed on an ultra reversed-phase C-18 column (5 µm 250 x 2.1 mm, Restek, Bellefonte, PA) with a binary gradient delivered by an Agilent binary pump (Hewlett Packard, CA). Solvent A was CH$_3$CN and solvent B was CHCl$_3$. A stepwise gradient with 80-40% A in B. Reproduced with permission from (93).

Hydroperoxy derivatives of linoleic acid (86). Molecular species containing combinations of one unmodified acid and two [9-oxo]nonanoic or two hydroperoxy acids were also present in significant amounts in peroxidized corn oil. The core aldehydes were identified as the DNPH derivatives, with the 1-(9-oxo)nonanoyl-2,3-diamitoyl-sn-glycerol giving a major ion at $m/z$ 901 ([M-1]$^-$ for DNPH) on reversed-phase LC/TSI-MS. A minor ion at $m/z$ 961 was attributed to an acetate adduct [M+59]$^+$ and another one at $m/z$ 983 was assigned to the deprotonated adduct of sodium acetate [M+82]-1$^-$ . A reversed phase LC/TSI-MS spectrum of 1,2-di-(9-oxo)nonanoyl-3-palmitoyl-sn-glycerol gave an
Figure 9. Total negative ion elution profile of the DNPH derivatives of rapidly oxidized sunflower oil TAGs and full mass spectrum averaged over the acylglycerol elution range. Peaks are identified as clusters in the elution profile and identified on basis of subsequent analyses as follows: cluster 1, trialdehydes; cluster 2, core aldehyde trihydroxides or epoxydihydroxides; cluster 3, core aldehyde dihydroxides or epoxyhydroxides; cluster 4, core aldehyde hydroperoxides or epoxyhydroxides; cluster 5, corealdehyde hydroperoxides; cluster 6, TBHP adducts of core aldehydes; cluster 7, TBHP adducts of core aldehydes; cluster 8, core aldehydes; cluster 9, core aldehydes. The LC/ESI-MS analyses were performed on a reversed phase HPLC column (Supelcosil LC-18, 250 x 4.6 mm ID) using a linear gradient of 20-80% 2-propanol in MeOH (0.85 ml/min) in 30 min. The column effluent was admitted to Hewlett-Packard Model 5989A quadrupole mass spectrometer interfaced with a nebulizer assisted ESI source. DNPH derivatives of TAG core aldehydes were determined as [M-H] ions in the negative ion mode. Reproduced with permission from (102).

$m/z$ 997 ([M-1- for DNPH) as the sole significant ion. The structures of the minor secondary products analyzed after derivatization were consistent with known oxidative degradation products of fatty acid hydroperoxides. In the glycerol ester form, both geometric and chiral isomers appear, which are subject to appropriate chromatographic resolution.

Sjovall et al. (102) also investigated the formation of TAG core aldehydes during rapid oxidation of corn and sunflower oils with tert-BOOH/Fe$^{2+}$. The core aldehydes were isolated as DNPH derivatives by preparative TLC and identified by reversed phase HPLC with on-line ESI-MS and by reference standards. Using the reversed phase HPLC system developed earlier (47), a total of 113 species of TAG core aldehydes were specifically identified, accounting for 32-53% of the DNPH-reactive material of high molecular weight, representing 25-33% of the total oxidation products. The major core aldehyde species (50-60% of total TAG core aldehydes) were the mono [9-oxo]nonanoyl- and mono[12-oxo]-9,10-epoxy dodecenoyl- or [12-oxo]-9-hydroxy-10,11-dodecenoyl-DAGs (Figure 9). The peaks possess symmetrical shapes, but are resolved into two or more components based on the relative proportions of the regio- and geometric isomer content.

The above reversed phase LC/ESI-MS system has since been used to demonstrate significant formation of core aldehydes during autoxidation of sunflower oil (103). Figure 10 shows the total negative ion current profile (A) of a stored sample of sunflower seed oil, along with the full mass spectrum (B) as obtained by LC/ESI-MS for the DNPH derivatives of core aldehydes. The DPNH derivatives of the core aldehydes were resolved by reverse phase HPLC on a Supelcosil LC-18 column. The instrument was a Hewlett-Packard Model 1050 Liquid chromatograph with UV detector (358 nm). The aldehydes were identified by LC-MS using a Hewlett-Packard Model 1090 HPLC interfaced with a nebulizer assisted ESI source connected to a Hewlett-Packard Model 5989A single quadrupole mass spectrometer. For improved ionization,
Figure 10. Total negative ion current profile of DNPH-derivatized core aldehyde containing TAGs of autoxidized (prolonged storage) sunflower seed oil as obtained by LC/ESI-MS (A), and the full mass spectrum (B) averaged over the core aldehyde elution range (5-25 min). The HPLC peaks are labeled as the major calculated neutral species, and the tentative identities of the masses are given as nominal mass of [M+DNPH]- are given in figure. The LC/ESI-MS conditions were as given in Figure 9. Reproduced with permission from (103).

ammonia/2-propanol (2%) was added at a post-column flow rate of 1.25 ml/min. DNPH derivatives were detected as [M-H]- ions in negative ion mode. The masses of the major DNPH derivatives of the core aldehydes range from 946 to 1006 amu as indicated by the labeling of the major ions making up each peak. These ions represent mainly the C9:0 and C12:1 core aldehydes of the major sunflower TAGs, which have been produced on storage at ambient and below ambient temperatures. The peaks were identified on basis of chromatographic and mass spectrometric evidence, which included ESI/CID-MS. In contrast to the samples oxidized by tert-BOOH, the autoxidized samples of sunflower oil contained both 12:1 and 13:2 core aldehydes. Under the selected experimental conditions the single ion mass chromatograms of the DNPH derivatives of the mono-aldehydes representing sn-2- and sn-1(3)-isomers were fully or partially resolved. Sjovall et al. (103) provided extensive tabulations of the o xo-TAGs of autoxidized sunflower oil. Quantitatively the core aldehydes made up 2-12 g/kg of oil by UV detection and 2-9 g/kg of oil by ESI-MS detection, whereas the hydroperoxides measured in the unreduced state by HPLC with ELSD were estimated at 200 g/kg after 18 days of autoxidation. Using these methods, Sjovall et al. (100) reported preliminary identification of mixed lipid ester hydroperoxides and core aldehydes, including C4-core aldehydes, from autooxidized TAGs of Baltic herring oil.

The presence of TAG containing chain-shortened aldehydes among the peroxidation products of vegetable oils has been demonstrated using APCI by Byrdwell et al. (104,105). Byrdwell and Neff (104) reported the formation of chain breakage products formed from the loss of a volatile short chain fragment (C10H18) from OOO subjected to frying temperatures. A protonated molecular ion at m/z 747.7 appeared along with an acylium ion, RCO+, at m/z 127.0. Other chain shortened OOO species appeared as a result of losing C6 (m/z 493.4), C9 (m/z 479.4), C10 (m/z 465.3), C11 (m/z 451.3) and C12 (m/z 437.3) volatile short chain fragments, which correspond to the core aldehydes.
anticipated from the chain cleavage of the various monohydroperoxides of OOO by Kuksis et al. (86) and Sjoval et al. (47) in the tert-butyl hydroperoxide oxidation products of both synthetic and natural TAGs containing oleic acid residues. These fragments were not simply formed in the APCI system. Byrdwell and Neff (106) have since demonstrated the advantages of a dual parallel ESI and APCI-MS system for the analysis of TAG oxidation products.

Steenhorst-Slikkerver et al. (107) analyzed the nonvolatile lipid oxidation products in vegetable oils by normal phase LC/MS. This resulted in a separation into classes of TAG oxidation products, such as epoxy-, oxo-, hydroperoxy-, hydroxy- and “2½ glycerides”, which were identified by SIM. LC-MS was performed on an HP 1100 LC/MS, which was operated using a binary high pressure pump, an auto-injector, a thermostated column and a diode-array detector. When these non-volatile lipid oxidation products were analyzed by reversed phase HPLC, the various species present were separated according to class: [OOH-TAG, hydroxy-TAG (OH-TAG), epoxy-TAG, etc.] and size [OOH-18:2/18:2/18:2, OOH18:1/18:2/18:2, OOH-18:1/18:1/18:2, etc.] (87). For the analysis of oxidation products originating from a single polyunsaturated parent TAG species, the reversed phase HPLC yielded excellent separations, while oxidation products of mixed TAGs gave complex chromatograms.

The polyunsaturated fatty acids 20:4n6, 20:5n3 and 22:6n3 are predominantly incorporated into glycerolphospholipids, but 11-18% of 22:6n3 may be found to be taken up into neutral lipid pools of the brain (108). These acids in their various ester forms would be anticipated to be subject to the oxidative influences demonstrated for the polyunsaturated glycerolphospholipids (43). However, no specific reports of isoprostane or neuroprostane containing TAGs would appear to have been reported, although prostaglandin esters of arachidonoylglycerol are known (44). Likewise, the 18:3n-3 containing TAGs of plant tissues would be anticipated to yield dinoprostane-containing acylglycerols, which also have not been reported, although the corresponding glycerolphospholipids are known (109).

Recent studies by Suomela et al. (110) have identified oxidized and chain shortened TAG molecules in pig chylomicrons. Figure 11 shows ion chromatograms obtained for the oxidized TAGs in pig chylomicrons. Postulated molecular structures (excluding regioisomers) are shown in the chromatograms. The products consisted of TAG molecules with a single hydroxyl, epoxy, keto or aldehyde function. On the basis of peak intensities, the hydroxides were the most abundant. The aldehydes are anticipated to be subject to the oxidative influences demonstrated for the polyunsaturated fatty acid moiety of cholesteryl esters is known to promote the oxidation of the cholesteryl ring. The oxidation of both free and esterified cholesterol has been extensively studied and reviewed by Smith (111, 112).

4.3.1. Primary products

Cholesterol autoxidation proceeds by initial formation of a carbon-centered radical at the allylic C7 site, with subsequent reaction of the radical with oxygen to yield a 3beta-hydroxycholesterol 5-ene-7-peroxy radical that is stabilized by abstraction of hydrogen from another substrate molecule, yielding the epimeric 7-hydroperoxides as stable products. A second carbon-centered radical is detected in solid cholesterol and identified as the tertiary C25 radical. Upon reaction with oxygen it yields the corresponding C25-peroxyl radical, from which 3beta-hydroxycholesterol 5-ene-25-hydroperoxide is derived by hydrogen abstraction. In addition to the autoxidation of cholesterol about the delta2-double bond, free cholesterol is autoxidized in solid state and in solution by a separate process at the 3beta-hydroxyl group, yielding hydrogen peroxide and the delta6'-5-ketone, which in turn is subject to rearrangement to the isomeric delta6'-3-ketone and to autoxidation by oxygen to yield epimeric 6alpha- and 6beta-hydroperoxycholest-4-en-3-ones.

Cholesterol hydroperoxides, including 3beta-hydroxy-5alpha-cholest-6-ene-5-hydroperoxide, 3beta-hydroxycholesterol-4-ene-5alpha-hydroperoxide, and 3beta-hydroxycholesterol-4-ene-6beta-hydroperoxide are formed in singlet excited oxygen-mediated reactions (113, 114).

For the less complex samples, TLC and HPLC serve well for isolation of individual oxysterols. Sevianian and McLeod (115), Malavasi et al. (54), Sevianian et al. (116) and Caboni et al. (52) described conventional TLC methods for the isolation of the 7-hydroperoxy and 7-hydroxycholesterol without resolution of epimers. More recently, Korytowski et al. (117) and Kriska et al. (118) have reported the TLC resolution of epimeric oxocholosteryl using high performance TLC plates (HPTLC). Figure 12 shows HPTLC-profile of [3H]cholesterol oxidation products in 5alpha-hydroperoxide-primed liposomes treated with iron/ascorbate (117). Extracted lipids were chromatographed on a silica gel HPTLC plate, using benzene/ethanol acetate (1:1, v/v) as the mobile phase. The separated radio-labeled species were detected and quantified by phosphorimaging. Oxocholesterol assignments were based on locations of authentic standards run separately or in spiked samples.

To the early workers, capillary GLC of oxosterol TMS ethers coupled to EI or CI-MS provided the most effective resolution, identification, and quantification of oxosterols. Thus, Park and Addis (119) and Park et al. (120) reported the use of a fused-silica capillary GLC column in combination with a Kratos MS25 mass spectrometer (Ramsey, NJ) to separate and confirm the identity of the TMS derivatives of standard oxosterols. One or more of the characteristic ions for M, M-15 [M-CH3], M-90 [M-TMS], M-105 [M-TMS-
Figure 11. Ion chromatograms showing oxidized TAG molecules of pig chylomicrons. Postulated molecular structures (excluding regioisomers) are given in Figure 11. (A) TAG of 54 acyl carbons with a hydroxy, a keto, or an epoxy group attached to a fatty acid; (B) TAG of 45 carbons (originally 54 carbons) with a FA core aldehyde (ALD) (as 2,4-dinitrophenylhydrazine derivative). HPLC separations were obtained with a Discovery HS C18 column (250 mm x 4.6 mm ID, Supelco) and a linear gradient of 20-80% 2-propanol in MeOH in 20 min. Fifteen percent of the column effluent was fed to a Finnegan MAT TSQ 700 triple quadrupole mass spectrometer equipped with nebulizer assisted ESI interface. Full mass spectra were taken in negative (m/z 600-1200) and positive (m/z 450-1100) ionization mode.

The nature of the possible peroxides obtained during copper peroxidation of LDL cholesterol have been described by Malavasi et al. (54), who also reviews the previous work on cholesterol oxidation. Analysis of the oxidation products by TLC and MS allowed to establish that 7alpha- and 7beta-hydroperoxycholest-5-en-3beta-ol (7alpha-OOH and 7beta-OOH) are largely prevalent among the oxosterols at early times of oxidation. The concentration of 7-hydroperoxycholest-5-en-3beta-ol decreased with oxidation time with a concomitant increase of cholest-5-en-3beta,.7alpha-diol (7alpha-OH), cholest-5-en-3beta,.7beta-diol (7beta-OH) cholesta-3,5-dien-7-one (CD) and cholest-5-en-3beta-ol-7-one (7CO). After 24 h oxidation a minor component of the LDL was cholestan-3beta-ol-5,6-oxide (EP). The presence of 7alpha- and 7beta- isomers of 7-OOH in the biological extracts was verified by reduction with NaBH₄. Figure 13 shows the GC/MS separation and identification of the oxocholesterols as the TMS ethers following NaBH₄ reduction (54)

Dzelotovic et al. (55) developed an isotope dilution-MS method for the determination of nine cholesterol oxidation products in human plasma. For GC-MS the sterols were converted to TMS ethers and the GC-
Figure 12. HPTLC-Piprofile of [14C]ChOX in 5alpha-OOH primed liposomes treated with iron/ascorbate. Timed samples using liposomes composed of POPC/[14C]Ch/5alpha-OOH (1.0/0.75/0.05 by vol), 1.0 mM AH−, and 1.0 microM Fe(HQ)3. Extracted lipids were chromatographed on a silica gel HPTLC plate, using benzene/EtAc (1:1, v/v) as the mobile phase. Radiolabeled species were detected and quantified by phosphorimaging. Incubation times were as indicated. ChOX assignments were based on locations of authentic standards run separately or in spiked samples. O, origin; F, solvent front; Rf values were as follows: 0.61 (Ch); 0.44 (7beta-OOH); 0.40 (7alpha-OOH); 0.34, (5,6-epoxide); 0.21 (7beta-OH); 0.16 (7alpha-OH). Sample load per lane was 55 nanomol total lipid; 12 nCi total radioactivity. Reproduced with permission from (117).

Figure 13. Total ion current GC-MS analysis of TMS derivatives of the oxosterol fraction of LDL incubated with 20 microM Cu2+ for 24 h. Peaks: A, cholest-5-en-3beta,7alpha-diol; B, cholesterol; C, cholesta-3,5-dien-7-one; D, cholest-5-en-3beta,7beta-diol; E, tentatively identified as cholestan-3beta-ol-5,6-oxide; , unknown; G, cholest-5-en-3beta-ol-7-one. GC-MS analyses were performed using HP5970 mass detector (Hewlett-Packard) connected with a HP5890 gas chromatograph on a 30 m SPB1 capillary column (0.22 mm ID and 0.12 microm film thickness, Supelco). The injector and transfer line temperatures were 265 °C and 280 °C, respectively. The samples were trimethylsilylated and injected at 150 °C and the oven temperature programmed from to 300 °C at the rate of 20 °C/min. The peaks were identified by reference to standards. Reproduced with permission from (54).
Figure 14. Ion chromatograms from an analysis of a typical plasma sample. The pairs of peaks correspond to deuterated internal standard (solid line), and unlabeled compound broken line. The compounds were eluted in the following order: I, \( \text{7alpha-hydroxycholesterol} \); II, \( \text{7beta-hydroxycholesterol} \); III, \( \text{cholesterol-5beta,6beta-epoxide} \); IV, \( \text{cholesterol-5alpha,6alpha-epoxide} \); V, \( \text{cholestane-3beta,5alpha,6beta-triol} \); VI, \( \text{24-hydroxycholesterol} \); VII, \( \text{25-hydroxycholesterol} \); VIII, \( \text{7-oxocholesterol} \); and IX, \( \text{27-hydroxycholesterol} \). The ions monitored for the different compounds are given in the text. The inset shows part of the ion chromatograms including peaks of \( \text{24-} \) and \( \text{25-hydroxycholesterol} \) and \( \text{7-oxocholesterol} \) in horizontal magnification. Reproduced with permission from (55).

MS was performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph equipped with an HP-5MS capillary column, connected to an HP 5972 mass selective detector and HP 7673A automatic sample injector. The mass spectrometer was operated in the selected ion monitoring mode, and two or four ions were detected at the same time. The cholesterol oxidation products determined were \( \text{cholesterol-5ene-3beta-7alpha-diol} \), \( \text{cholesten-5ene-3beta-7beta-diol} \) (\( \text{7alpha-} \) and \( \text{7beta-hydroxycholesterol} \), respectively), \( \text{3beta-hydroxycholest-5-en-7-one (7-oxocholesterol)} \), \( \text{5,6alpha-epoxy-5alpha-cholestan-3beta-ol} \) (cholesterol-\( \text{5beta,6beta-epoxide} \)), \( \text{5,6beta-epoxy-5beta-cholestan-3beta-ol} \) (cholesterol-\( \text{5beta,6beta-epoxide} \)), \( \text{cholestane-3beta,5alpha,6beta-triol} \), \( \text{cholestane-3beta,5alpha,6beta-triol} \), \( \text{24-hydroxycholesterol, 416, 17.59; 24-hydroxycholesterol, 413, 17.63; [\( \text{2H6} \)]-25-hydroxycholesterol, 134, 17.86; 25-hydroxycholesterol, 131, 17.89; [\( \text{2H6} \)]-7-oxocholesterol, 478, 17.95; 7-oxocholesterol, 472, 18.04; [\( \text{2H3} \)]-27-hydroxycholesterol, 461, 18.75; 27-hydroxycholesterol, 456, 18.82. The electron ionization energy was 70 eV. The 5,6-oxygenated products were present mainly unesterified while the other oxidation products were mostly in esterified form (Figure 14).

In a parallel study, Patel et al. (121) used the method of Dzelotovic et al. (55) to compare the results of oxidation of LDL by peroxynitrite, myoglobin and copper on the basis of the pattern of formation of nine oxosterols using isotope dilution mass spectrometry. To determine the fate of \( \text{7alpha-hydroperoxycholesterol} \) during analysis of free oxysterols (i.e., when omitting alkaline hydrolysis), cholesterol was converted into \( \text{5alpha-hydroperoxycholest-7-en-3beta-ol} \) by photosensitized oxidation (114). The \( \text{5alpha-hydroperoxycholest-7-en-3beta-ol} \) was allowed to isomerizes to \( \text{7alpha-hydroperoxycholesterol} \) by standing overnight in chloroform and subsequently purified by TLC. The pattern of sterols obtained showed, that it was formed independent of the nature of the oxidant, and that \( \text{7-oxocholesterol} \) and \( \text{7beta-hydroxycholesterol} \) are formed in substantial quantities with all three reagents. The yields of products identified decreased in the order \( \text{7-oxocholesterol} \)...
Oxolipids

> 7beta-hydroxycholesterol > 7alpha-hydroxycholesterol > 5,6beta-epoxycholesterol > 5,6alpha-epoxycholesterol except in the case of peroxynitrite in which case a higher yield of 5,6beta-epoxycholesterol was seen. Endogenous alpha-tocopherol in LDL or supplementation with BHT prevented oxysterol formation.

Breuer and Bjorkhem (122) reported the simultaneous quantification of several cholesterol autoxidation and monohydroxylation products by deuterium-labeled internal standards. Serum samples were dried under vacuum and the oxosterols converted to TMS ethers. Combined GC-MS was performed with Hewlett-Packard 5970 quadrupole mass spectrometer with Hewlett-Packard 5890 capillary gas chromatograph and Hewlett-Packard 7698A automatic sample injector. EI ionization was applied at 70 eV. Selective ion monitoring was performed by cycling the quadrupole mass filter between different m/z at a rate of 3.7 cycles/sec. Quantitative estimates were obtained for 7alpha-hydroxycholesterol, cholesterol-5beta,6beta-epoxide, cholesterol-5alpha,6alpha-epoxide, 7-oxocholesterol, cholestanetriol, 25-hydroxycholesterol, and 26-hydroxycholesterol.

Hodis et al. (123) coupled Hewlett-Packard 5985A quadrupole mass spectrometer to HP 5840 capillary gas chromatograph to separate and identify the TMS ethers of oxidized cholesterol by chemical ionization in the presence of ammonia. The method was applied to determine the relationship between plasma and arterial wall oxysterols, plasma and aortic tissue from 7 New Zealand White rabbits fed high cholesterol diet for 6 weeks. Normalcholesterolemic plasma and aortic tissue contained low levels of cholest-5-ene-3beta,7alpha,17alpha-diol, cholesta-3,5-diene-7-one, 5,6-epoxy-3alpha-ol, cholest-5-ene-3beta,7alpha-diol, and 5alpha-cholestane-3beta,5,6beta-triol, while hypercholesterolemic plasma and atherosclerotic aorta contained significantly higher levels of these compounds. Furthermore, 6,6beta-epoxy-5alpha-cholestan-3beta-ol not found in normocholesterolemic plasma or aortic tissue was present in substantial amounts in both hypercholesterolemic plasma and atherosclerotic aortic tissue.

Csalán et al. (124) reported an HPLC method for quantification of cholesterol and four of its major oxidation products (7-ketocholesterol, 7alpha-hydroxycholesterol, 7beta-hydroxycholesterol, and 25-hydroxycholesterol) in muscle and liver tissues. The separations were performed on a normal phase column. The identities of the oxosterols were confirmed by MS of standards. Sevanian et al. (116) compared the application of GC-MS and LC-MS for analysis of plasma cholesterol oxidation products. The GC-MS was performed with Shimadzu GC-14A gas chromatograph equipped with a 30 m DB-1 capillary column coupled to Finnigan MAT 50 quadrupole mass spectrometer. For LC-MS analysis using a TSI interface, a Hewlett-Packard HPLC 1090A was used which was fitted with a 60 mm x 4.6 mm ID Hewlett-Packard RP-18 Hypersil column. The eluent was delivered by means of a TSI interface to a Hewlett-Packard 5988A mass spectrometer. Analysis of human and rabbit plasma samples identified 7beta-hydroxycholesterol, cholesterol-5alpha,6alpha-epoxide, and cholesterol-5beta,6beta-epoxide as commonly occurring components. The latter two compounds were dramatically increased in hypercholesterolemic samples and were present in approximately equal amounts in the free cholesterol and cholesteryl ester fractions. Despite the readily measurable levels of the above cholesterol oxides, there was no evidence for cholesterol-7-hydroperoxides associated with plasma free cholesterol. Sevanian et al. (116) used normal phase HPLC for isolation of total 7-hydroperoxysterols and epimeric 7alpha- and 7beta-hydroxycholesterols.

Bachowski et al. (113) reported the HPLC profiles of the 6beta-hydroperoxy-, 5alpha-hydroperoxy-, 7alpha-hydroperoxy-7beta-OH cholesterols as recovered from photoperoxided L120 leukemia cell lipid extracts. Definitive identification of the lipid hydroperoxides species by GC-MS or high resolution NMR was not possible due to low yields, peroxide microheterogeneity, and the presence of co-eluting, non-peroxide contaminants.

Caboni et al. (52) reported HPLC separation of non-derivatized cholesterol oxidation products using Spherisorb S5 CN columns. Effective resolution was obtained (in order of increasing retention time) for standard 5alpha,6alpha-epoxy, 5beta,6beta-epoxy + 4beta-OH, 20alpha-OH, 7-keto, 25-OH, 19-OH, 7alpha-OH, 7beta-OH, 7alpha-OOH, 7beta-OOH cholesterols and a triol. The first six peaks were found to be present in the unsaponifiable fraction of egg yolk powder. The separated HPLC peaks were identified by GC-MS with ion trap MS (Finnigan MAT ITYS40, San Jose, CA). Caboni et al. (52) used normal phase HPLC for effective resolution of 7alpha- and 7beta-hydroperoxycholesterols and 7alpha- and 7beta-hydroxycholesterols. In both instances the oxosterol peaks were identified by GC/MS following reduction of hydroperoxides and preparation of TMS ethers.

Plat et al. (57) determined the oxidized plant sterols in human serum and lipid infusions by combining GC-MS with deuterated internal standards. The GC-MS analysis was performed on the TMS ethers using a capillary RTX5MS column coupled to a GCQ-plus ion trap (Thermoquest CE instrument (Engelbach, Germany). Analysis was carried out in a single ion monitoring mode. Plat et al. (57) have given the GC-MS retention times and m/z values for the detection of plant sterols, oxophytosterols, cholesterol and oxosterols as TMS derivatives. Both campesterol and sitosterol oxidation products were found. The steryl ester samples were saponified prior to oxosterol analysis. 7-Ketositosterol, 7beta-hydroxyisositosterol, 5alpha,6alpha-epoxyisositosterol, 3beta,5alpha,6beta-sitostanetriol, and probably also 7alpha-hydroxyisosterol were present in markedly elevated concentrations in serum from phytosterolemic patients only.

4.3.2. Secondary products

The thermal decomposition of initially formed hydroperoxides affords a particularly complex...
mixture of cholesterol autoxidation products (111,112). The hydroperoxide decomposition proceeds by peroxide bond homolysis, putatively yielding the corresponding oxyl radical whose stabilization may occur by hydrogen abstraction, giving the corresponding alcohol (formal reduction), and by beta-scission (of primary or secondary hydroperoxides), yielding the corresponding carbonyl derivative (formal dehydration). Homolysis of the peroxide bond of 7-hydroxycholesterol yields the corresponding 3beta-hydroxycholesterol-5-en-7-oxyl radicals, from which the 3beta,7-diol and 7-ketone are derived. The 7-hydroperoxides are also reduced to the corresponding 3beta,7-diol in reaction with the delta5-double bond of cholesterol, in which the isomeric 5,6-epoxides results. The epimeric 6-hydroperoxides yield the corresponding epimeric 6alpha- and 6beta-hydroxycholesterol-4-en-3-ones and cholest-4-ene-3,6-dione as products. Oxosterols may be further oxidized, for example, the 3beta,5alpha,5beta-triol is oxidized to 3beta,5dihydroxy-5alpha-cholestan-6-one and the 3beta,7alpha-diol (via epoxidation and hydration) to 5alpha-cholestane-3beta,5,6beta-triol, which can be used to distinguish them from enzymatically created during manipulations, and, second, the 7-hydroperoxide decomposition proceeds by peroxide bond scissions of carbon-carbon bonds of the steroid tetracyclic nucleus have not been observed in autoxidation. The tertiary 25-hydroperoxide yields the 3beta,25-diol by formal reduction, but also 3beta-hydroxy-27-norcholest-5-en-25-one, chol-5-ene-3beta-ol, and pregn-5-en-3beta-ol by beta-scissions of the C25/C27, C24/C25 and C20/C22 bonds, respectively.

There are two analytical problems that need to be considered in all cases. First, oxosterols are inevitably created during manipulations, and, second, the 7-hydroperoxides and 7-ketone may suffer decompositions in analysis. Smith (56,112) has discussed the formation of hydroperoxides and 7-ketone may suffer decompositions in autoxidation mixtures containing about 15 sterols, including the familiar secondary oxidation products: cholesta-3,5-dien-7-one, 25-hydroxycholesterol, 7-ketocholesterol, 7beta-hydroxycholesterol, 7alpha-hydroxycholesterol, 7alpha,5beta-dihydroxy-5alpha-cholestan-6-one, and 5alpha-cholestane-3beta,5,6beta-triol (in order of decreasing mobility). However, it was obvious that all autoxidation mixtures contained components that remained unresolved by this method. HPTLC, which is capable of much greater resolution (see above) has not yet been applied to the resolution of the secondary oxidation products of cholesterol.

Bascoul et al. (126) used capillary GLC and TLC on Chromarods with flame ionization detection to identify and quantify the oxosterols in tallows heated under deep frying conditions. Of the original cholesterol 25% was converted into unidentified products during cooking and partly transformed into 3beta,5,6beta-trihydroxy-5alpha-cholestan-7alpha-hydroxy-, 7beta-hydroxy-, 7-ketocholesterol, 7-oxocholesterol, 7-oxocholestan-3,5-diene and cholesterol epoxides. The identity of the unknowns in some cases was confirmed by GC-MS analysis of the TMS ethers and methoximes on a LKB 2091 at 780 eV. A clear separation was obtained for 7-oxocholesterol and all of cholesterol epoxides with alpha- and beta-configurations and syn- and anti-methoxime isomers of cholestanone.

Park et al. (120) used GC/MS for a kinetic evaluation of 3beta-hydroxycholesterol-5-en-7-one stability during saponification, which had previously led to a controversy. It was shown, that during room temperature saponification the loss of 7-ketocholesterol was negligible, while at 75 °C for 30 min caused extensive destruction leaving only 31% of the original 7-ketocholesterol. Careri et al. (127) evaluated HPLC with PB-MS for analysis of cholesterol secondary oxidation products. Both normal phase and reversed phase chromatographic methods making use of narrow bore columns were set up for simultaneous separation of these compounds. PB-MS spectra were recorded in the EI, and positive or negative CI modes and the corresponding spectral data recorded. Among the many derivatives obtained by further degradation of hydroperoxides in cholesterol-containing foods, 25-hydroxycholesterol, cholestan-3beta,5alpha,6beta-triol, 5alpha,6alpha-epoxy-cholesterol, 5,6beta-epoxy-cholesterol, 7alpha-hydroxycholesterol, 7-ketocholesterol and cholesta-4,6-dien-3-one have been identified as major products. Using the reversed phase LC/PB-MS method detection limits at the low nanogram level were achieved for all the analytes.

Osada et al. (53) used reversed phase HPLC with on-line ESI-MS to identify the oxosterols resolved by reversed phase HPLC. This analysis was simultaneously performed by post-column method after UV detection by connection of photodiode-array detector and mass spectrometer. After passing through a photo-diode-array detector, oxidized cholesterol derivative standard mixture was subjected to LC/ESI-MS analysis. The column effluent was mixed post-column with 0.5% ammonium hydroxide and positive ionization spectra were taken in the m/z range 300-500. Single ion mass chromatograms were retrieved from the LC/ESI-MS data (Figure 15). The characteristic ions of each oxidized cholesterol derivative were as follows: 25-hydroxycholesterol, m/z 367, 385, 413, 425 and 441; 26-hydroxycholesterol, 367, 385, 413, 425, and 441; cholestanetriol, 367, 385, 413, 438 and 441; 7alpha-hydroxycholesterol, 367, 385, 413, 425, and 441; cholesta-4,6-dien-3-one, 387, 405 and 421; and 4,6-cholestadien-3-one, 383, 405 and 421.

4.4. Oxysterol esters
Cholesterol esters represent a high proportion of bound polyunsaturated fatty acids, which are subject to
Figure 15. Reversed phase LC/EI-MS spectra of standards of secondary oxidation of cholesterol. Peak identification: Peak 1, 25-hydroxycholesterol; Peak 2, 26-hydroxycholesterol; Peak 3, cholestanetriol; Peak 4, 7alpha-hydroxycholesterol; Peak 5, 7beta-hydroxycholesterol; Peak 6, 7-ketocholesterol; Peak 7, 5beta-epoxycholesterol; Peak 8, 5alpha-epoxycholesterol; Peak 9, 4,6-cholestadien-3-one; Peak 10, 3,5-cholestadien-7-one; Peak 11, cholesterol; Peak 12, 20alpha-hydroxycholesterol; Peak 13, 6-ketocholesterol; Peak 14, 19-hydroxycholesterol; Peak 15, 5alpha-cholestan-3,6-diene. HPLC conditions: column, Supelcosil LC-18 (25 cm x 4.6 mm, ID, 5µm); mobile phase, isocratic MeOH/MeCN (60:40, v/v) over 30 min; detector, UV from 190 to 290 nm. ESI-MS, Hewlett-Packard Model 5988B single quadrupole mass spectrometer. Positive ionization spectra were taken over the mass range 300 to 500. Post-column addition of 0.5% NH₄OH at 0.3 ml/min. Reproduced with permission from (53).
peroxidation. Because the sterol ring also becomes oxidized, it is necessary to employ chemical or enzymatic hydrolysis for structural identification of the oxidation products even when MS/MS is preceded by chromatography. Both primary and secondary products of oxidation are produced at the level of the cholesterol ring and the fatty acid moiety.

4.4.1. Primary products

Kamido et al. (30) used GC-MS to identify the ring oxidized cholesterol moiety released by cholesteryl ester hydrolase from oxidized cholesteryl palmitate and oleate. The GC/MS analyses of the TMS derivatives of the oxidation products were performed as described previously for the oxidation products of unesterified cholesterol (25). In the present instance, 30 m x 0.32 mm ID capillary columns coated with DB-5 were used with temperature programming. The carrier gas was hydrogen at 6 psi. The 7alpha- and 7beta-hydroxy cholesteroles gave strong [M-90]+, [M-90-15]+ as well as other minor ions characteristic of the TMS ethers. An [M]+ ion at m/z 382 was also obtained for cholestadien-7-one, which was assumed to be a minor product of dehydration of 7-ketocholesterol in the gas chromatograph (128). The unknown peaks yielded strong ions corresponding to m/z 384, which suggested that they were isobaric dehydration products of oxocholesterol. The mass spectra of the oxosterols agreed with the corresponding spectra recorded in the literature for 7-ketocholesterol (129), 3,5-cholestadien-7-one (130), and the 7alpha- and 7beta-hydroxy cholesteryl di-TMS derivatives (131). Reduction of 7-ketocholesterol oleate by NaBH4 gave the corresponding 7alpha- and 7beta-hydroxy cholesteryl stearates, which upon saponification and trimethylsilylation gave the anticipated GLC results.

Because the bond dissociation energy of a bis allylic carbon-hydrogen bond in the polyunsaturated fatty chain is substantially lower than that of an allylic carbon-hydrogen bond in cholesterol, the peroxidation of cholesteryl linoleate and arachidonate yields predominantly linoleate and arachidonate peroxides. Cholesteryl linoleate hydroperoxide and cholesteryl arachidonate hydroperoxide (and their isomers) have been characterized as products of LDL oxidation by GC-MS of the oxo fatty acid moieties (132, 133). Kritharides et al. (134) used reversed phase HPLC and isotropic solvent system of CH3CN/2-propanol (44:56:2, by vol) for the identification of intact cholesteryl ester oxidation products and accomplished it without reducing hydroperoxide groups to the more stable hydroxides. Individual unknown compounds were collected manually following separation by an HPLC system. Analysis was performed on a Finnigan TSQ 700 mass spectrometer using CI with ammonia as the ionization reagent gas. Cholesteryl linoleate hydroperoxide standard (Mr 680) produced major ions at mass 698 (M+18)4, 682, and 664. It was likely that decomposition of the hydroperoxyl group to the hydroxyl (thus producing cholesteryl linoleate hydroxide, Mr 664) occurred during handling and heating, producing ions of cholesteryl linoleate hydroxide 682 (M+18)3 and 664. Both the cholesteryl linoleate standard and the early oxidation product of LDL yielded thermal decomposition ions consistent with cholesterol (m/z 386, 369 [MH-H2O]+, and 368 [M-H2O]+).

Kenar et al. (15) identified and quantified the regioisomeric hydroperoxides of cholesteryl linoleate exposed to aqueous radicals generated from the thermal labile azo compound 2,3’-azobis(2-aminodimopropane) dihydrochloride, or a free radical initiator di-tet-butyl hyponitrite (DTBN) synthesized in the laboratory. In addition, they established methodology based on HPLC to directly assay for the regioisomeric cholesteryl linoleate hydroperoxides (Ch18:2-OOH) and alcohols in oxidized LDL and HDL. Figure 16A shows a typical HPLC analysis (234 nm detection) of the Ch18:2-OOH isomeric mixture. Peaks I and II are clearly resolved, while Peaks III and IV partially overlap. Figure 16B shows the separation of the alcohols obtained from the isolated hydroperoxides by triphenylphosphine. 1H-NMR and chemical transformation allowed the identification of the four alcohols as follows: Two sets of essentially identical 1H NMR spectra represented, presumably, the cis,trans isomers 1 and 3, while the trans,trans isomers 2 and 4 formed another set. The isolated Ch18:2-OHs were transesterified with the use of NaOMe to yield the hydroxy linoleate esters, which had been previously characterized by GC-MS.

Folcik and Cathcart (135) used HPLC to compare the products obtained by copper and soybean 15-lipoxygenase oxidation of LDL cholesteryl linoleate. Reversed phase HPLC was performed with Microsorb C18 columns using a gradient of MeCN and 2-propanol. Effective separations were obtained for free cholesterol (peak 1); cholesteryl HPODE (peak 2); cholesteryl HODE (peak 3), trioleoylglycerol (peak 4) and cholesteryl linoleate (peak 5), as well as other unidentified peaks. Under these conditions, cholesteryl 13- and 9-HPODE (or HODE) and the cis,trans and trans,trans isomers of each positional isomer are not separated by the employed solvent system. The identity of the monohydroxylinoleic acid metabolites was determined by reference to radioactive standards and was confirmed by GC-MS. Later Folcik et al. (136) used reversed phase HPLC to isolate the cholesteryl HPODE and cholesteryl HODE peaks using the previously described method in a comparison to the human 15-lipoxygenase oxidation products and primary oxidation products of cholesteryl linoleate recovered from atherosclerotic lesions. After reduction, saponification, and methylation, the cholesteryl linoleate oxidation products were subjected to a chiral phase HPLC column chromatography with (R)-N-(3,5-dinitrobenzoyl)-alpha-phenylglycine ionically linked to aminopropyl residues on silica gel as described by Kuhn et al (7). The chiral stationary phase yielded quantitative estimates of the S and R isomers in each fraction. On the basis of a somewhat higher contribution of hydroperoxy-octadecadienoate (13(S)-HPODE) it was concluded that lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques (136).

Kuhn et al. (137) used chiral packings [(R )-N-(3,5-dinitrobenzoyl)-alpha-phenylglycine ionically linked to aminopropyl residues on silica gel] as the chiral
stationary phase and 0.5% isopropanol as the mobile phase (7) for the resolution of the stereoisomers of oxidized cholesteryl linoleate hydroperoxides (following reduction and saponification). There was no evidence of stereospecific oxidation of cholesteryl linoleate in advanced human atherosclerotic plaques.

Dzelotovic et al. (56) demonstrated that in copper oxidized LDL a significant consumption of both free and esterified cholesterol occurred. In fact, esterified cholesterol was consumed more readily than free cholesterol. Most of the arachidonic acid and linoleic acid in the LDL particle was consumed within their first few hours of incubation with copper. In contrast, oleic acid was consumed slowly throughout the entire incubation period.

After 24 hours of oxidation approximately half of the oleic acid remained. It had been previously shown that copper oxidation leads to formation of monohydroxylated fatty acids that may be derived from oleic acid (133). Esterified cholesterol was consumed to a larger extent than free cholesterol. The 7-oxygenated products dominated over the 5-oxygenated and the difference was slightly more marked in case of lipoxygenase incubation. As shown by TLC, cholesterol hydroperoxides appeared already after 1 h of copper incubation. The cholesteryl hydroperoxides were formed before other oxosterols could be detected by GC-MS, which is in accordance with the general view that the hydroperoxides are the initial products of cholesterol autoxidation (111,112).

Figure 16. HPLC chromatograms (0.5% 2-propanol in hexane, 234 nm) from the autoxidation of cholesteryl linoleate with DTNB at 37°C. (A) Isomeric Cholesteryl 18:2-OOHs: I, Cholesteryl 13-hydroperoxyoctadecaa-cis-9,trans-11-dienoate; II, Cholesteryl 13-hydroperoxyoctadecaa-trans-9,trans-11-dienoate; III, Cholesteryl 9-hydroperoxyoctadaecaa-trans-10,cis-12-dienoate; IV, Cholesteryl 9-hydroperoxyoctadecaa-trans-10,trans-12-dienoate; V, Cholesteryl 13-hydroxyoctadecaa-cis-9,trans-11 dienoate; VI, Cholesteryl 13-hydroxyoctadecaa-trans-9,trans-11dienoate; VII, Cholesteryl 9-hydroxyoctadecaa-trans-10,cis-12-dienoate; VIII, cholesteryl 9-hydroxyoctadecaa-trans-10, trans-12-dienoate. Reproduced with permission from (15).
Oxolipids

In confirmation of an earlier observation, Mashima et al (22) reported nanomolar levels of all free radical-mediated oxidation products of cholesteryl linolate, 13ZE, 13EE, 9EZ, and 9-EE forms in blood plasma, whereas the 13ZE-isomer resulting from enzymatic 15-lipoxygenase oxidation was not evident as a major product. The oxidation of cholesteryl linolate and cholesteryl eicosadienoate was performed in 2-propanol with 100 mM AMVN [2,2'-azobis(2,4-dimethylvaleronitrile)] at 37 °C. The oxidation products were purified by reversed phase HPLC (Supelcosil LC-18, 4.6 x 250 mm) with MeOH/2-propanol as mobile phase. The regioisomeric composition of the cholesteryl linolate hydroxides was determined by normal phase HPLC sing hexane/2-propanol (1000:5, v/v) as the mobile phase. The HPLC elution profiles for the hydroperoxides and hydroxides or cholesteryl linolate, cholesteryl eicosadienoate (external control) and cholesteryl linolate were determined by GC-MS (138).

More recent studies (139) have detected significant autooxidation of the cholesterol ring along with the oxidation of the linoleic acid ester chain, which preceded it. This contrasts with ozonization of the cholesterol ring prior to ozonization of the linoleic acid moiety of the cholesteryl ester (5,140). Herrera et al (139) used a combination of TLC, HPLC and LC/ESI-MS to determine the time course of copper peroxidation of both sterol and fatty acid moieties of cholesteryl linolate. With ammonia as the reagent gas, a good quasi-molecular ion [M+NH4]+ and ions diagnostic for both sterol and fatty acid moieties were obtained. Both C9 and C14-hydroperoxides of reference cholesteryl linolate prepared by copper oxidation were readily separated and detected. The cholesteryl linolate oxidation products were first resolved by TLC on the basis of polarity. In a parallel study, the o xo-cholesteryl ester mixture was reacted with DNPH and the reaction products separated on basis of polarity in another TLC system. Subsequently, each TLC band was subjected to reversed phase HPLC with on-line mass spectrometric monitoring.

4.4.2. Secondary products

Using reversed phase HPLC and CI-MS with ammonia, Kritharides et al. (135) noted the appearance of products of advanced oxidation of cholesteryl esters, when the incubation of LDL of cupric salts was extended to 24 and 48 hours. These products were not detectable by chemiluminescence. Four of these compounds (Peaks A, B, C and D) chosen for their prominence and reproducibility were identified as 7-ketocholesterol (Peak A, Mr 400), based on mass 418 and 401 ([M+18]+ and [MH]+, respectively); unknown (Peak B, Mr 698), based on a fragment ion indicating cholesterol (ion mass 386); cholesteryl linolate hydroxide (Peak C, Mr 664), based on mass 682 and 665 ([M+18]+ and [MH]+, respectively); unknown (Peak D) initially produced major ions of mass 640, 656 and 658, while later in the same analysis ions of mass 402, 383, and 367 appeared. This suggested thermal decomposition to oxosterols, most probably 7-ketocholesterol or 7-hydroxy cholesterol. In a follow-up study, Brown et al. (141) used the methods of Kritharides et al. (134) to further resolve and identify the copper-oxidized free and esterified cholesterol of LDL. The oxocholesterol moieties of oxocholesterol esters were identified by GC-MS of the TMS ethers following saponification of the ester fraction. This approach allowed, in case of the most abundant oxoester, 7-ketocholesterol, a distinction between esters with oxidized or unoxidized fatty acyl chains. An investigation of the time course of copper-catalyzed LDL oxidation showed that free cholesterol was most resistant, followed by cholesteryl palmitate, whereas cholesteryl docosahexaenoate was the most susceptible to oxidation. The oxosterols formed are the same as seen with cholesteryl autooxidation and hence similar mechanisms of sterol oxidation is warranted. An initial formation of cholesteryl linolate hydroperoxide is followed by the formation of the epimeric 7-hydroperoxides, and a propagation radical. The peroxide bond may then undergo cleavage yielding the corresponding cholesterol 7-alkoxy radicals which are subject to hydrogen abstraction to form 7-ketocholesterol. There remained some unidentified oxidized cholesteryl esters, which probably included the core aldehydes characterized by Kamido et al. (25).

Havrilla et al. (17) point out that lipid peroxidation of polyunsaturated fatty acid esters of cholesterol leads to a complex mixture of hydroperoxides and cyclic peroxides, including dozens of diastereomers and regioisomers, which can be successfully analyzed by coordination ion-spray mass spectrometry (CIS-MS). Silver ion forms readily detected Ag+ adducts of peroxides and hydroperoxides. These ions, observed at [M+107] and [M+109], undergo fragmentation typical of hydroperoxides, and cyclic peroxides. Furthermore, silver ion CIS-MS can be coupled to normal phase HPLC by post-column addition of AgBF4, allowing the use of powerful techniques such as SIM and SRM. This coupling has permitted, for the first time, the assignment of defined structures, after separation, by conversion of the cholesteryl ester to the corresponding methyl esters and comparison with samples previously characterized. For on-line LC-MS separation and analysis of the cholesteryl ester hydroperoxides, the peroxidic oxidation products were isolated from the unoxidized lipid by semipreparative chromatography. The oxidized fractions for cholesteryl linolate and cholesteryl arachidonate were collected, concentrated, and analyzed by LC/CIS-MS. Furthermore, each of the concentrated fractions was rechromatographed to give pure cholesteryl arachidonate alcohols, which were examined by 1H NMR. Specifically identified were: cholesteryl 15-hydroxy-5(Z),8(Z),11(Z),13(E)-eicosatetraenoate; cholesteryl 12-hydroxy-5(Z),8(Z),10(E),14(Z)-eicosatetraenoate; cholesteryl 11-hydroxy-5(Z),8(Z),12(E),14(Z)-eicosatetraenoate; cholesteryl 9-hydroxy-5(Z),7(E),11(Z),14(Z)-eicosatetraenoate; cholesteryl 8-hydroxy-5(Z),9(E),11(Z),14(Z)-eicosatetraenoate; and cholesteryl 5-hydroxy-6(E),8(Z),11(Z),14(Z)-eicosatetraenoate formed in the presence of a good hydrogen atom donor, such as 1,4-cyclohexadiene. The product mixture became much more complex when cyclohexadiene was not used. Peroxyl radicals derived from cholesteryl arachidonate can undergo peroxy radical cyclization in competition with hydrogen atom abstraction, and cyclization can lead to a host of different products.
Yin et al. (38) have identified a novel class of peroxides (dioxolane-isoprostanes) having a bicyclic endoperoxide moiety characteristic of the isoprostanes and a dioxolane peroxide functionality in the same molecule formed from in vitro autooxidation of cholesteryl arachidonate. CIS-MS was accomplished as previously described (17). Various other MS techniques (such as SIM and SRM) were applied to characterize these new peroxides. Figure 19 shows the mixture of products obtained from oxidation of 12-HPETE cholesterol as analyzed by Ag⁺ CIS-MS (direct solvent infusion) (38).

In addition to isomerization, cyclization, oxidation and reduction, the hydroperoxides of polyunsaturated cholesteryl esters decompose into aldehydes by homolytic scission of the two carbon bonds on either side of the hydroperoxy group (9). A cleavage of the cholesteryl ester of a fatty acid hydroperoxide would then yield a water-soluble aldehyde derived from the methyl terminal of the unsaturated fatty acid and a lipid soluble core aldehyde still containing the cholesterol moiety. Kamido et al. (25) prepared synthetic standards of cholesteryl [5-oxo]valerates and [9-oxo]nonanoates, which were isolated by TLC, purified by HPLC, and characterized by GC-MS with ammonia and LC-MS in NCI as the DNPH derivatives. GC-MS spectra were obtained for the free aldehydes and the methoxime (MOX/TMS) derivatives (figures not shown). These standards were used to identify the core aldehydes generated during copper oxidation of low density lipoproteins (142). The core aldehydes were isolated as the dinitrophenylhydrazones, and were identified by reversed phase HPLC with mass spectrometry. The major components were the C₇-C₁₀ alkanoyl esters of cholesterol and 7-ketocholesterol, and accounted for 1-2% of the cholesteryl linoleate and arachidonate consumed. Figure 20 shows the reversed-phase LC-MS total NCI current profile of the DNPH derivatives of the core aldehydes derived from copper oxidation of low density lipoprotein along with the molecular ion plots for the species (128). The major components were cholesteryl [5-oxo]valerate (m/z 664) and cholesteryl [9-oxo]nonanoate (m/z 720), with much smaller amounts of cholesteryl [4-oxo]butyrate (m/z 650), cholesteryl [6-oxo]hexanoate (m/z 678), [7-oxo]heptanoate (m/z 692) and [10-oxo]decanoate (m/z 734), as indicated by the ion intensities listed on the left-hand side of the figure. The mass spectra of the cholesteryl [9-oxo]nonanoate and [5-oxo]valerate corresponded exactly to those of synthetic standards (25). The method was later applied to the identification of cholesteryl core aldehydes among the copper peroxidation products human plasma LDL and HDL with similar results (61). Interestingly, there were no unsaturated core aldehydes recognized, although monounsaturated derivatives would have been anticipated at least as intermediates in the oxidation process.

Kawai et al. (143) have recently confirmed that peroxidation of LDL with Cu⁺ produces [9-oxo]nonanoyloxycholesterol (9-ONC) and [5-oxo]valeroyloxycholesterol as the major oxidized cholesteryl esters. It was observed that after a peak at 12 h, the there was a significant decrease in the cholesteryl ester core

**Oxilipids**

**Figure 17.** Chromatograms of cholesteryl arachidonate oxidation mixture: (A) UV detection at 234 nm; (B) HPLC/CIS-MS in selected ion monitoring mode for m/z = 875 – 591; (C) HPLC-CIS-MS in selected reaction mode for m/z = 875 – 657. Normal phase HPLC was carried out using two tandem Beckman Ultrasound narrow bore 5 microm silica columns (2.0 mm x 25 cm) operated in isocratic mode with 0.35% 2-propanol in hexane. Column effluent was passed through a UV detector a 234 nm. An Upchurch high pressure mixing tee was connected next in series for the post-column addition of the silver salts. The silver tetrafluoroborate (AgBF₄) solution (0.25 mM in 2-propanol) was added via a Harvard pump. A section f the PEEK tubing allowed for complexation of the silver to the lipid while delivering the complex to the mass spectrometer (Finnigan TSQ-7000 triple quadrupole instrument equipped with API ESI source outfitted with a 100 m deactivated fused Si capillary. Reproduced with permission from (17).

Figure 17 shows SRM chromatograms, which provide detailed information about the serial structures that are present in the product mixture (17).

Yin et al (59) have used the methodology of Havrilla et al (17) to examine prostaglandin bicyclic endoperoxides from the peroxidation of cholesteryl arachidonate by LC-MS and GC-MS techniques. All four possible types (I-IV) of bicyclic endoperoxides were found starting from different regioisomeric hydroperoxides of cholesteryl arachidonate. Yin et al. (59) have also determined the stereochemistry of Type IV bicyclic endoperoxides by conversion to PFB ester and TMS ether, and by comparison with synthetic standards using GC-MS. All eight possible diastereomers of the derivatized isoprostanes were observed and were separated by GLC. Figure 18 shows a CID analysis of Type IV bicyclic endoperoxides of cholesteryl arachidonate using direct liquid infusion with Ag⁺CIS-MS. The structure of the compounds has been confirmed by independent synthesis. On the basis of the free radical mechanisms of the transformation, it was concluded that only 12- and 8-peroxyl radicals (those leading to 12-HPETE and 8-HPETE) of arachidonate can form these new peroxides.
aldehydes, which was accompanied by an increase in the 7-
ketocholesterol ester core aldehydes and by an increase in
apoB-bound cholesterol. To detect the protein bound core
aldehyde, a monoclonal antibody was raised against an
antibody against 9-ONC–modified protein. It was found
that it extensively recognized protein-bound core
aldehydes. The core aldehyde-protein complex was
subjected to alkaline hydrolysis and the released (bound)
cholesterol and 7-ketocholesterol were identified by HPLC.

Monounsaturated core aldehydes of cholesteryl esters were
identified, however, among the tert-butyl hydroperoxide
oxidation products of cholesteryl linoleate and arachidonate
(30). Reversed-phase LC-MS showed

Figure 18. CID of Type IV bicyclic endoperoxides of cholesteryl arachidonate. (A) The oxidation products of cholesteryl 15-
hydroperoxyeicosatetraenoate (HPETE) detected by direct liquid infusion with Ag⁺CIS-MS; (B) CID of Type IV bicyclic
endoperoxide (m/z 843). CIS-MS in both instances was performed as in Figure 17. Argon served as a collision gas. Reproduced
with permission from (59).
total negative ion current profiles indicating the presence of monounsaturated core aldehyde esters (as the DNPH derivatives) originating from the milder peroxidation of the cholesteryl linoleate and arachidonate. The major components in this fraction are the cholesteryl [11-oxo]undecanote (m/z 746) and cholesteryl [12-oxo]decanone (m/z 780) and cholesteryl [9-oxo]nonanote (m/z 720). Similarly, monounsaturated core aldehyde esters were obtained from tert-butylhydroperoxidation of cholesteryl arachidonate. Here the major components were the cholesteryl [5-oxo]valerate (m/z 664) and cholesteryl [7-oxo]heptanote (m/z 690), with much smaller amounts of cholesteryl [4-oxo]butyrate (m/z 650), cholesteryl [6-oxo]hexanote (m/z 676), [9-oxo]nonadecanote (m/z 716), and [10-oxo]deca-diecenote (m/z 730). Kamido et al. (61) and Hoppe et al. (28) detected cholesteryl ester core aldehydes in human atherosclerotic plaques using essentially identical LC/MS methods, including preparation of the DNPH derivatives. The core aldehydes produced by the oxidation of fatty acids esterified to cholesterol, have been analyzed comprehensively (144). The most abundant core aldehyde from this source was found to be 9-oxononanoyl cholesterol, the oxidation end-product of linoleate, which is the most prominent polyunsaturated fatty acid in cholesteryl esters.

Hoppe et al. (28) demonstrated a Schiff base formation between cholesteryl [9-oxo]nonanote and $\varepsilon$-amino group of N-BOC-Lys (Figure 21). The free $\varepsilon$-amino group readily reacted with the core aldehyde to yield Schiff base. The base gave a correct molecular mass of m/z 769 [M+1]$^+$ on flow injection/ESI-MS at a capillary exit (CapEx) of 120V. The base was stabilized by NaBH$_4$ reduction with increased mass to m/z 771 and permitted chromatographic purification. Increasing the CapEx from 120 to 300V gave fragment ions supporting the Schiff base structure of this compound yielding fragment ions anticipated from a loss of the tert-butyl group [M-57]$^+$ at m/z 715, [M-N-BOC-Lys]$^+$ at m/z 525, and [M-N-BOC-Lys+(oxononanote]$^+$ at m/z 369 (cholesterol ring). Hoppe et al. (28) also obtained evidence for a covalent interaction of $[^3]$Hcholesteryl ester core aldehydes with serum proteins. The formation of detergent resistant complexes between lipoprotein-deficient serum and core aldehydes was further confirmed by SDS/PAGE, suggesting a covalent interaction. Kawai et al. (143) demonstrated that the cholesteryl and 7-
Figure 20. Reversed phase LC-MS of the DNPH derivatives of (A) cholesteryl ester and (B) 7-keto cholesteryl ester core aldehydes in negative ion mode. Peak identification (A): C_4 (m/z 650), [4-oxo]butyroyl cholesterol; C_5 (m/z 664), [5-oxo]-valeroyl cholesterol; C_6 (m/z 678), [6-oxo]hexaenoyl cholesterol; C_7 (m/z 692), [7-oxo]heptanoyl cholesterol; C_8 (m/z 706), [8-oxo]octanoylcholesterol; C_9 (m/z 720), [9-oxo]nonanoylcholesterol; C_{10} (m/z 734), [10-oxo]decanoylcholesterol. Peak identification (B): m/z 580, DNPH of 7-keto cholesterol; C_4 (m/z 664), DNPH of [4-oxo]butyroyl 7-ketocholesterol; C_5 (m/z 678), DNPH of [5-oxo]valeroyl 7-ketocholesterol; C_6 (m/z 692), DNPH of [6-oxo]hexanoyl 7-ketocholesterol; C_7 (m/z 706), DNPH of [7-oxo]heptanoyl 7-ketocholesterol; C_8 (m/z 720), DNPH of [8-oxo]octanoyl 7-ketocholesterol; C_9 (m/z 734), DNPH of [9-oxo]nonanoyl 7-ketocholesterol; C_{10} (m/z 748), DNPH of [9-Oxo]nonanoyl 7-ketocholesterol; Tl, total ion current; 600-800, ion current over the mass range 600-800 amu. DNPH derivatives of core aldehydes were separated by reversed phase HPLC using Supelcosil LC-18 column (250 x 4.6 mm) using MeCN/2-propanol (4:1, v/v) or a linear gradient of 30-90% CH_3CN in CH_3CN as the eluting system. NCI spectra were taken every 5s over the entire chromatogram in the mass range 200-900 amu. Reproduced with permission from (128).
Figure 21. Demonstration of Schiff base formation between cholesteryl 9-oxononanoate and the epsilon-amino group of N-BOC-Lys. (A) Total positive ion current of NaCNBH₃ reduced base as obtained by PLC/ESI/MS at CapEx voltage of 120. (B) Full mass spectrum at CapEx =120V averaged over the entire peak eluted in (A). (C) Full mass spectrum at CapEx =300 V averaged over the entire peak eluted in (A). Ion identification: m/z 771, [M+1]+; m/z 788, [M+NH₄]+; m/z 715, [M-tert-butyl+1]+; m/z 845, [M+57+18]+; m/z 525, [M-N-BOC-Lys]+; m/z 369, cholesterol ring; m/z 303, reduced Schiff base of N-BOC-Lys and 9-oxononanoate. HPLC analysis of the reduced reaction product of lysine and cholesteryl ester core aldehyde were performed on Spherisorb columns (3 µm, 100 mm X 4.6 mm ID, Alltech) installed in a Hewlett-Packard model 1090 liquid chromatograph connected to a Hewlett-Packard 5988B Quadrupole mass spectrometer, and equipped with a nebulizer assisted ESI interface. The column was eluted with a linear gradient of 100% solvent system C (CHCl₃/MeOH/30% NH₄OH (80:19.5:0.5 by vol) by changing to 100% solvent system D (CHCl₃/MeOH/30% NH₄OH (60:34.5:5.0:0.5 by vol) in 14 min, followed by 100 system D for 10 min. Reproduced with permission from (28).酮基胆甾醇酯核心醛在腹膜和LDL apoB（图22）。如图所示，在LC-MS的末尾产物在21 min时显示了一个假分子离子峰m/z 832.1 [M+H]+，对应于9-oxo]nonanoylcholesterol-lysine adduct。此外，通过进一步的分析，我们可以发现[9-oxo]nonanoylcholesterol的损失比其他任何氨基酸都高。为了检测蛋白质中产生的C₉醛，我们通过酶联反应法和免疫测定法检测了LPS的免疫活性。结果表明，酮基胆甾醇酯核心醛在LDL中的形成可能代表了脂质氧化过程的共同机制。在这一点上，有必要提到Herrera et al (139)已经证明了7-ketocholesterol和7-hydroperoxycholesterol的9-oxo]nonanoyl esters，这些醛基团也可能会与蛋白质结合。由于7-hydroperoxycholesterol被认为是氧化LDL的主要细胞毒性物质（145），因此，酮基胆甾醇酯核心醛可能代表了一个感兴趣的目标。
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Figure 22. Reaction of Nα-benzoyl-glycyl-lysine (10 mM) with [9-oxo]nonanoylcholesterol (5 mM) in 50 mM sodium phosphate buffer (pH 7.4, 37 °C, 4 hours). The products were stabilized with NaCNBH₃ and characterized by LC-MS.

Top, selected ion current chromatograms obtained from LC-MS analysis monitored with m/z 832; Middle, HPLC profile of the reaction mixture; Bottom, mass spectrum of the peak eluted at 21 min in the HPLC chromatogram. Reproduced with permission from (143).

Finally, it may be noted that in addition to autoxidation and enzymatic oxidation, oxosterols and oxosterol esters are also produced by ozonization, which has been recently reviewed elsewhere (5).

5. SIGNIFICANCE

The significance of lipidomics of neutral oxolipids arises from their biological activity (146-148). Although this subject is not part of the present review, it is pertinent to call attention to several recent studies where metabolic interactions between oxolipids and amino acids, polypeptides and proteins have been demonstrated and specific biological consequences observed. Thus, 7β-hydroperoxycholesterol as well as 7-oxo- and 7-hydroxycholesterol [145] have been proposed to be the primary cytotoxins in oxidized LDL. Oxosterols are potent signaling lipids that directly bind liver X receptors and a subset of oxysterol binding protein related proteins (149). Staprans et al (150) report that oxidized cholesterol in the diet accelerates the development of atherosclerosis in LDL receptor and apolipoprotein E-deficient mice. Suomela et al. (110) reports the presence of lipid ester hydroperoxides and core aldehydes in chylomicrons of pigs fed peroxidized fat. Van Heek et al. (151) report that cholesteryl hydroperoxyoctadecadienoate from oxidized LDL inactivates platelet-derived growth factor.

Ravandi et al. (50) and Kurvinen et al. (96) have shown that glycerophospholipid and monoacylglycerol core aldehydes, respectively, react readily with amino acids and polypeptides. Hoppe et al (28) and Kawai et al (143) have demonstrated that cholesteryl and 7-ketocholesteryl ester core aldehydes bind to lysine and to LDL apoB. The results suggested that the binding of cholesteryl ester core aldehydes to LDL might represent the process common to the oxidative modification of lipoproteins. Amino acid and protein binding has also been reported (44) for the highly reactive gamma-ketoaldehydes (neuroketals) arising by peroxidation from the esters of docosahexaenoic acid. Interestingly, Sottero et al (152) reported that expression and synthesis of TGFβ is induced in macrophages by C₉ cholesteryl ester core aldehydes. Huber et al (153) reported that oxidized cholesteryl linoleates (including core aldehydes) stimulate endothelial cells to bind monocytes via the extracellular signal regulated kinase ½ pathway. Hartvigsen et al (98) identified peroxidation of 1-O-alkyl-2-(omega-oxo)acyl-sn-glycerols in shark liver oil and human milk as a potential source of PAF mimics and gamma-hydroxybutyric acid. The C₉ core aldehyde of both cholesterol and glycerol esters after reduction may be released as the neurologically active street drug GHB (154).

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