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Identification of oxidized phospholipids by electrospray ionization mass spectrometry and LC–MS using a QQLIT instrument

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ABSTRACT

Phospholipids are complex and varied biomolecules that are susceptible to lipid peroxidation after attack by free radicals or electrophilic oxidants and can yield a large number of different oxidation products. There are many available methods for detecting phospholipid oxidation products, but also various limitations and problems. Electrospray ionization mass spectrometry allows the simultaneous but specific analysis of multiple species with good sensitivity and has a further advantage that it can be coupled to liquid chromatography for separation of oxidation products. Here, we explain the principles of oxidized phospholipid analysis by electrospray mass spectrometry and describe fragmentation routines for surveying the structural properties of the analytes, in particular precursor ion and neutral loss scanning. These allow targeted detection of phospholipid headgroups and identification of phospholipids containing hydroperoxides and chlorine, as well as the detection of some individual oxidation products by their specific fragmentation patterns. We describe instrument protocols for carrying out these survey routines on a QTrap5500 mass spectrometer and also for interfacing with reverse-phase liquid chromatography. The article highlights critical aspects of the analysis as well as some limitations of the methodology.

Introduction

Oxidation of phospholipids occurs in a variety of physiological and pathological situations and has multitudinous effects on biological systems: the number of known biological and signaling effects of oxidized phospholipids is steadily increasing along with our understanding of their importance in mammalian cell function [1]. The early products of phospholipid peroxidation are hydroperoxides, which can rearrange to compounds containing epoxides, hydroxides, or ketones and isoprostane-like structures. Further oxidation and destabilization can result in cleavage of the oxidized fatty acyl chain to yield chain-shortened phospholipids and a corresponding nonesterified molecule. Methods for analyzing lipid peroxidation and phospholipid oxidation products have been available for nearly a century and vary from simple colorimetric assays to complex antibody- or technology-dependent procedures [2,3]. As a generalization, the quality and quantity of information is proportional to the complexity of the assay, although simple assays that measure a generic set of oxidation products also have their uses, for example, in rapid screening or when it is not necessary to discriminate between different oxidation products (occasionally a global measure of lipid damage is useful). If more specific analysis of individual products is desired, prior separation by chromatography is the best approach, of which HPLC offers the most flexibility for interfacing with various detection systems such as UV, fluorescence, electrochemical, chemiluminescence, or mass spectrometry.

Many assays depend on the reaction of the lipid oxidation product of interest with a chemical reagent to enable detection, for example, by the formation of a chromophore or fluorophore or by light emission. However, the assay reagents are not necessarily as specific as desired and may give misleading results. For example, there are a number of reagents that react with carbonyl groups, which as mentioned above are common end products of lipid peroxidation; examples include 2,4-dinitrophenylhydrazine, cysteamine, and cyclohexanedione. These will react with any carbonyl-containing products of lipid peroxidation, but also with other aldehydes and ketones that are not lipid-derived, including some carbohydrates. Thus specific identification of lipid peroxidation products depends on separation of the assay products before detection and use of standards prepared by reaction with known compounds, but even so it is sometimes not possible to resolve lipid peroxidation products from interfering compounds and identify them definitively. The thiobarbituric acid-reactive substances assay, which is commonly used for malondialdehyde, suffers from such limitations [4]. Chemiluminescence methods involving the reaction of hydroperoxides (from fatty acids, cholesterol, or phospholipids) postcolumn with luminol or isoluminol in the presence of microperoxidase are also frequently used, but equally depend on resolution quality and standards for identification of the exact species. Ultimately, any method that depends on a specific chemical modification is only as good as the

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specificity of the assay reagent. The same limitation theoretically applies to methods based on antibodies, such as the use of EO3 and EO6 antibodies that recognize 1-palmitoyl-2-(5′-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC)[1] adducts on LDL [5].

An advantage of mass-spectrometry-based methods is that they measure the mass-to charge ratio (m/z) of compounds, which represents a more selective although not unique characteristic compared to those described above. This means that several species that coelute during chromatography can nevertheless be identified. Gas chromatography–mass spectrometry has been the method of choice for analysis of fatty acids and isoprostanes [6], but is compatible only with volatile compounds, thus requiring chemical modification to hydrolyze phospholipids to fatty acids and derivatize these first to pentafluorobenzyl esters followed by formation of volatile trimethylsilyl ethers. This is a labor-intensive procedure with the corresponding danger of artifact introduction during the chemical reactions. In contrast, soft ionization mass spectrometry techniques such as electrospray ionization–mass spectrometry (ESI–MS) can be used for many ionizable species and interface ideally with liquid chromatography, so phospholipid extracts can be analyzed without further manipulation [3]. In analysis of phospholipid oxidation, mass spectrometry has the advantage that it is not dependent on functional group or reactivity, but a wide variety of oxidative modifications can be detected. Thus in addition to products of radical-induced peroxidation, it can also identify products of electrophilic oxidation, such as the formation of chlorinated lipids after attack with hypochlorous acid (HOCl) or nitrated products resulting from reactions with peroxynitrite or other reactive nitrogen species [3].

There is a wide variety of strategies for the application of ESI–MS to the analysis of phospholipid oxidation, as detailed in the next section, and the application of MS technology to studies of lipid oxidation in biological and biomedical science is rapidly expanding as researchers become aware of the potential of the approach and MS instruments become more accessible. The methodology is continually being developed and new applications are constantly emerging [7–9]. There are many reports on the application of ESI–MS or LC–MS to biological and clinical samples. The presence of fatty acid hydroperoxides derived from phospholipids has been observed in oxidatively stressed red blood cells [10] and phospholipid chlorohydrins were detected in LDL [11] using LC–MS. Several novel oxidation products of 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) were identified in

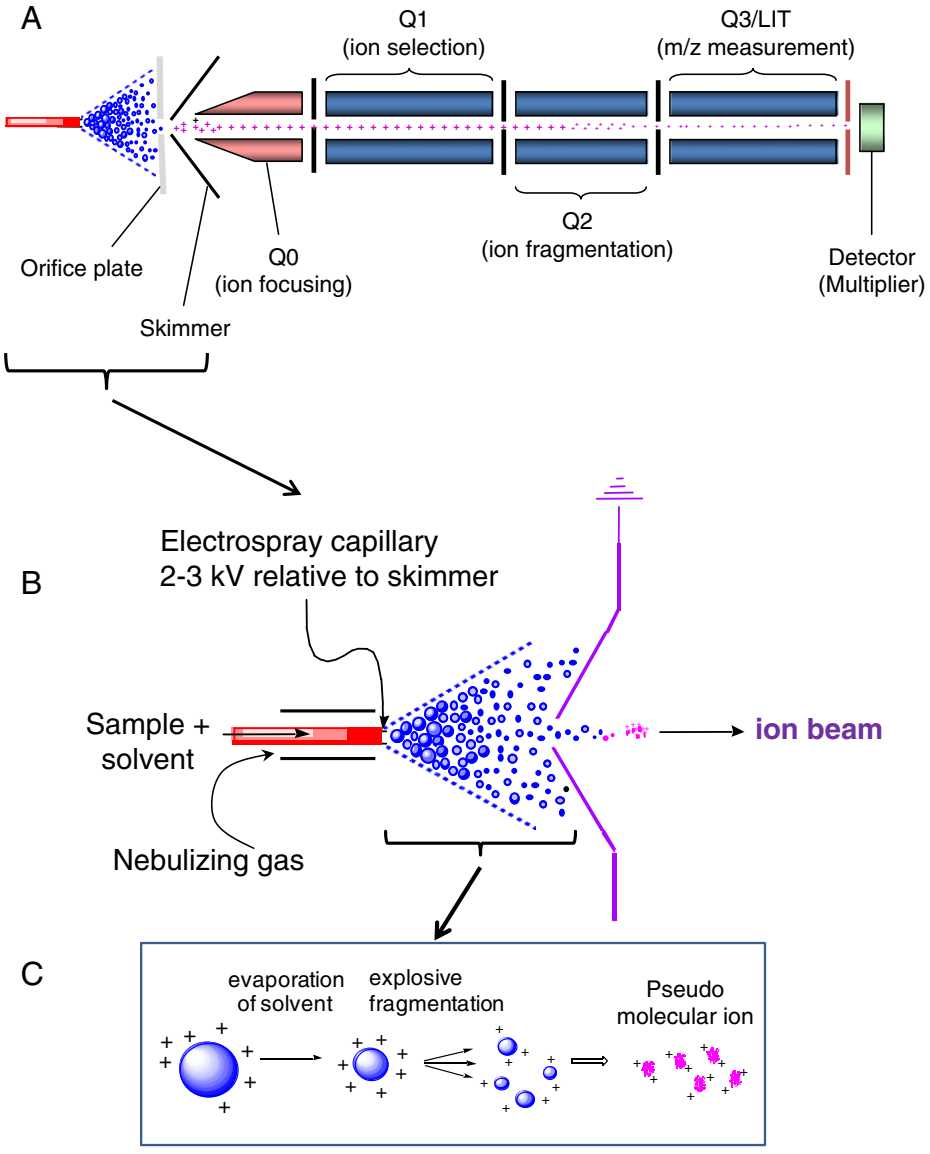

Fig. 1. Schematic diagram of an electrospray source showing formation of pseudo-molecular ions. (A) The geometry of a tandem or quadrupole–quadrupole–linear ion trap instrument. (B) The sample (in aqueous-organic solvent) is sprayed through a charged capillary and the solvent is evaporated by the sheath of nebulizing gas. (C) Pseudo-molecular ions form by explosive fragmentation as the droplet dries.

vitro and also in rabbit atherosclerotic lesions using MS^2 and other supporting techniques [12,13]. In human atherosclerotic tissue, a wide range of oxidized phosphatidylcholines were found, including hydroperoxides and hydroxides of phosphatidylcholine, as well as F_2-isoprostanes esterified to phosphatidylcholine and chain-shortened oxidized phospholipids containing aldehydes [14,15]. Chlorinated products have also been detected, using mass-spectrometry-based methods [16,17]. Most of these studies approached the detection and identification of oxidized phospholipids by selecting particular oxidized phospholipids of interest and then carrying out fragmentation to obtain structural information. A number of studies have also used multiple-reaction monitoring (MRM), which allows targeted detection of individual species only. This is commonly used for analysis of specific fatty acids in negative-ion mode [18,19] but can also be used in positive-ion mode with headgroup analysis [20]. This is a highly focused and sensitive method that is ideal when researchers are interested only in observing and quantifying certain predetermined species, rather than investigating the broader profile of phospholipid oxidation products. Intermediate between these approaches is the use of precursor ion scanning and neutral loss scanning, which allow certain types of phospholipid or oxidized products thereof to be targeted without specifying individual species. On the whole, these routines have been less exploited to date, but offer considerable potential [21,22].

The aim of this article is to provide straightforward protocols for ESI–MS, LC–MS, and scanning fragmentation experiments that can be followed by nonexperts who want to begin using mass spectrometry for the study of oxidized phospholipids. It is not a comprehensive and advanced guide for researchers who are already experts in the fields of lipidomics or oxidative lipidomics. Mass spectrometry is a complex technique and the number of experimental variations is almost as large as the number of groups with expertise in this field; many of these methods are equally good so long as attention is paid to critical experimental details. Likewise, there are different mass spectrometers and liquid chromatography systems available that enable the separation of oxPL from models and biological samples. Hence we have described methods for our instrument that work well, but have noted some possible variations together with important provisos. The article is focused on analysis of mammalian phospholipids oxidized in vitro or extracted from LDL or cells, but the principles can readily be applied to phospholipids from plants or microorganisms. It is important to note that detection of oxidized phospholipids in biological extracts is considerably more challenging that analysis of in vitro models, owing to greater complexity of lipid composition, low relative abundance of oxidized species, and issues with their stability in vivo.

Principles

Mass spectrometry analysis of phospholipids and oxidized phospholipids

Mass spectrometry measures the mass (or more accurately the *m/z*) of ions generated from molecules of interest and is also able to fragment the ions and measure the *m/z* of the product ions, which together allow the identification of many compounds. ESI–MS is a low-energy (often referred to as soft) ionization technique [23] that is ideal for easily ionizable biological molecules, as molecular ions can be formed in the source of the mass spectrometer without fragmentation [24]. The sample is introduced into the source through a charged capillary, as shown in Fig. 1, either by direct infusion of the sample using a syringe pump or by coupling to a liquid chromatography system; the latter allows complex mixtures to be separated before analysis in the mass spectrometer, which allows a deeper analysis and enables the analysis of isomeric compounds with the same mass (isobaric compounds).

Mass spectrometers can operate in positive- or negative-ionization mode. The polarity used is an important consideration as it affects the PLs that can be observed and the form in which they ionize [25,26]. Most phospholipids are zwitterionic and therefore need a counterion on one of the charge groups to give an overall positive or negative charge. In positive-ion mode phosphatidylcholines give the strongest signals as they have a constitutive positive charge on the headgroup, with the forms $[M]^+$ or $[M-H+Na]^+$ being the most common. As is common with the other phospholipids observed in positive-ion mode, the proton or sodium ion are associated with the phospho group in these species,

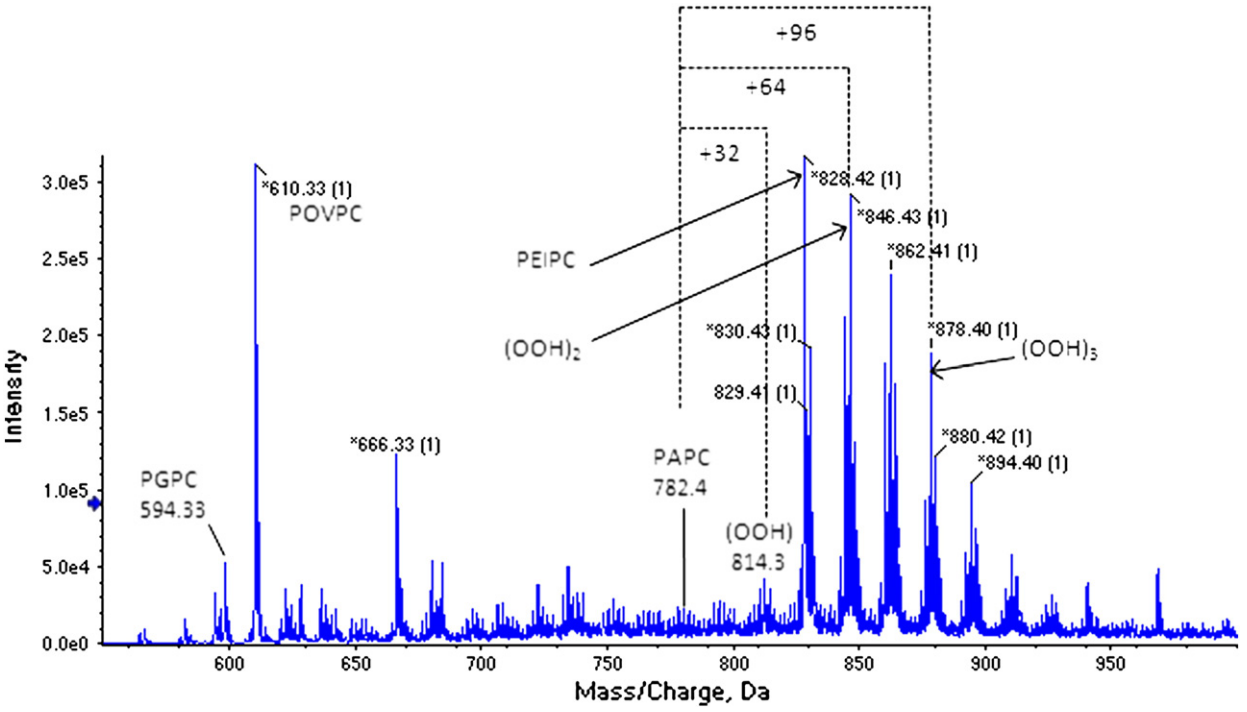

Fig. 2. EMS spectrum of autoxidized PAPC in + ve ion mode.

and other ions can also take their place, especially the alkali metals (Li and K) and ammonia. Phosphatidylethanolamines (PEs) can readily acquire a positive charge on the amine group to form $[M+H]^+$. Other PLs, including cardiolipin (diphosphatidylglycerol), phosphatidylserine (PS), and phosphatidylinositol, are observed more easily in negative-ion mode by deprotonation of the phosphate group ($[M-H]^-$) [25].

The first stage of phospholipid oxidation by oxygen radicals involves the incorporation of one or more dioxygen molecules into the fatty acid

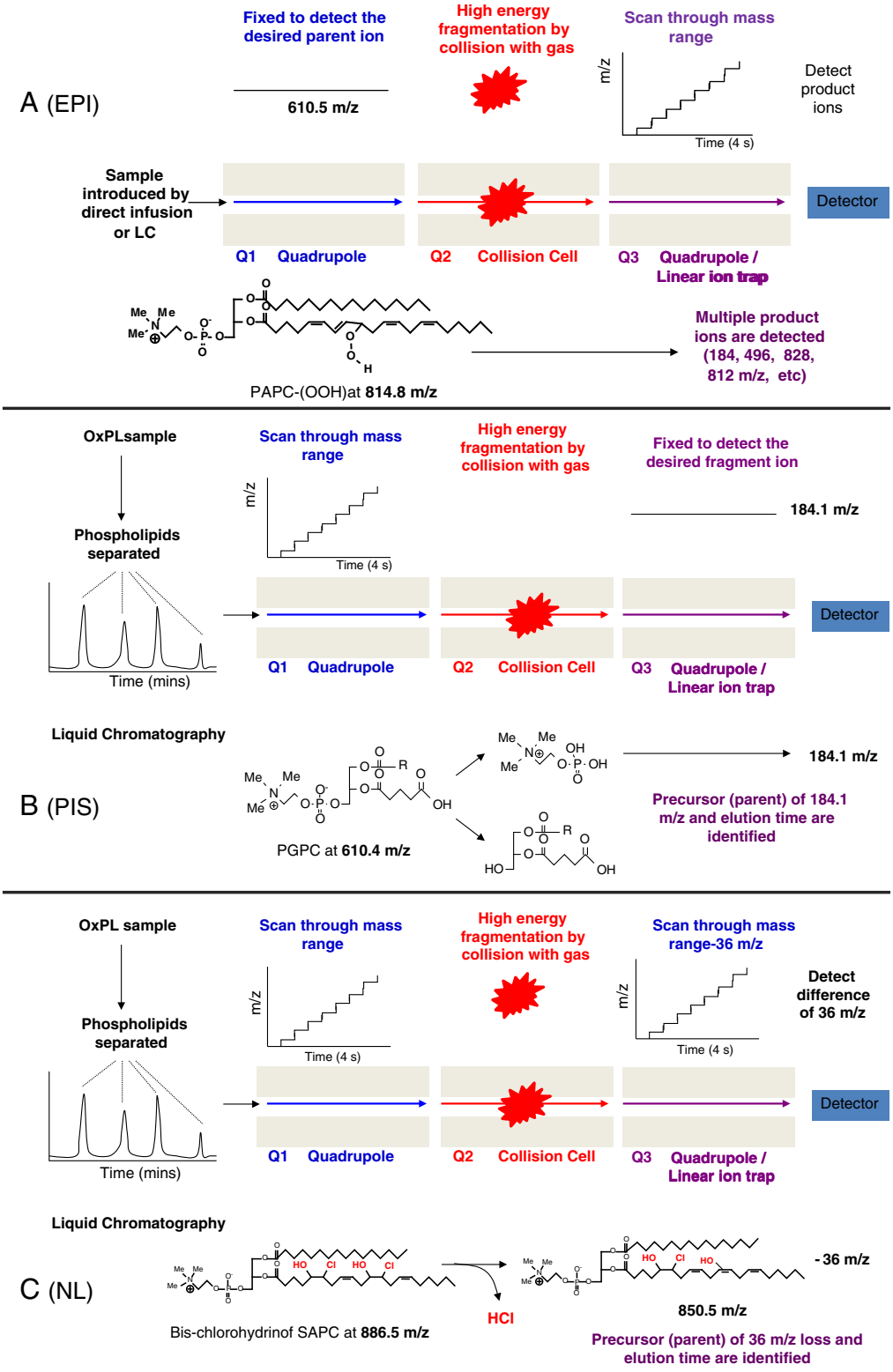

Fig. 3. Advanced MS routines for targeted detection of molecular species. (A) Product ion scanning (EPI) selects a specific molecular ion in Q1, fragments it in Q2, and scans to determine the *m/z* ratios of the product ions in Q3. (B) Precursor ion scanning mode scans through the mass range in Q1; at each step the molecular ions are fragmented in Q2, and Q3 is fixed to select the passage of a specific product ion, which can then be related to the precursor (parent) ion. (C) Neutral loss scanning mode scans both Q1 and Q3 simultaneously but with a fixed mass offset between them, so that precursor ions that fragment in Q2 by loss of a specific neutral fragment can be identified.

side chains to form hydroperoxides, which results in an increase in *m/z* by multiples of 32 Da [27]. Subsequent rearrangement, additional oxidation, and cleavage of the oxidized fatty acyl chain lead to further changes in the *m/z*. One of the great strengths of mass spectrometry is that a significant number of these oxidation products can be observed simultaneously in the mass spectrum (Fig. 2). Nonradical oxidation, for example, by HOCl or peroxynitrite ($ONOO^-$), results in the formation of other distinctive products, including chlorohydrins and nitrated lipids, and likewise alters their *m/z*[28,29]. If all the components in a mixture have different *m/z's*, they will give distinct signals in the spectrum. However, isobaric ions (with the same *m/z*) do occur, such as the oxidation product of a shorter chain fatty acid (monohydroperoxide of PLPC, *m/z* 790) and a native longer chain fatty acid (distearoyl-PC, *m/z* 790), and these are indistinguishable using their *m/z* alone by MS when using direct infusion; separation by chromatography or MS^2 fragmentation is required to distinguish the phospholipids in this case.

Separation of oxidized PLs by HPLC

Liquid chromatography interfaces ideally with ESI–MS, as the sample needs to be introduced in a liquid flow, and the mixture of aqueous and polar organic solvents is well suited to the generation of a good spray. Either normal-phase or reverse-phase columns can be used for separation of phospholipids: normal phase involves a silica stationary phase with nonpolar mobile phase and is most effective for separating phospholipid classes, but the solvents are less well suited to generating a good electrospray, whereas reverse phase involves a nonpolar capped silica stationary phase and a polar mobile phase, which separates phospholipids based on fatty acyl chain length and saturation and is better suited to integration with electrospray. A recently introduced method, hydrophilic interaction chromatography, is seeing increasing use in lipid analysis and is effectively a more robust form of normal-phase separation that uses solvents more compatible with mass spectrometry [30]. Reverse phase with C8 or C18 columns works best for identification of oxidized phospholipids, as these are more polar than native PLs and elute earlier from the column. Either isocratic or gradient elution can be used; typical mobile phases are a mixture of aqueous buffer with methanol or acetonitrile, although other solvents such as hexane or isopropanol are sometimes used as cosolvents. The solvent system usually contains an ion-pairing reagent for the chromatography, such as ammonium acetate, ammonium formate, or acetic or formic acid, to reduce the formation of mixed species with other counterions and to facilitate ionization. The choice depends on polarity of the ionization and which PL species are of interest. In this method, we use ammonium acetate, which we have found to be an effective ion-pairing reagent and is compatible with both positive- and negative-ion modes.

Fragmentation methods to identify oxidized PLs

An important strength of tandem mass spectrometry is the ability to select an ion, fragment it in the collision cell, and measure the *m/z* of the fragments. This can be used in a number of different ways to aid phospholipid analysis. Although the *m/z* and elution time are often sufficient to identify a phospholipid and its general structure with reasonable confidence, information on the distribution of fatty acyl chains and oxidation sites is desirable. This can be obtained by fragmenting the molecular ion in the collision cell of the mass spectrometer and recording a mass spectrum of the product ions.

There are several mass spectrometry routines that can be used to selectively identify ions containing specific motifs, based on varying the scans done in the two mass analyzers and how they are correlated, as illustrated in Fig. 3. A common approach (product ion analysis) involves selecting a molecular ion of interest in the first mass analyzer, fragmenting it by collision with gas in the collision cell, and scanning the fragments (product ions) in the second mass analyzer to obtain their *m/z* and information about the composition of the parent molecular ion, for example, the position of (un)modified acyl chain in the glycerol moiety, nature of the modification (functional group), and location of functional group within the carbon acyl chain [31]. This routine is useful for structural identification but it is time consuming to do this individually for all components of a complex mixture. Two powerful selective methods for identifying lipids containing specific structural components are precursor ion scanning and neutral loss scanning. Precursor ion scanning involves the identification of molecular species that give rise to a particular product ion after fragmentation. An example is that phosphatidylcholines fragment to give a phosphocholine moiety, which has a mass at *m/z* 184.1. If the second analyzer is set up to detect this ion and the first analyzer is scanned through the mass range, the *m/z's* of all the species that fragment to give ions at *m/z* 184 can be determined. This works well for molecular ions that fragment to yield a diagnostic or informative ion of the same polarity as the parent ion, but cannot easily be used, for example, to determine which phosphatidylcholines contain a particular fatty acid, as the latter is usually detected most effectively as a negatively charged ion. Neutral loss scanning works by scanning through the *m/z* range in both mass analyzers, but with a predetermined offset between them, so that ions that lose a certain mass on fragmentation can be detected. For example, phosphatidylethanolamines can fragment by loss of the phosphoethanolamine headgroup (141.1 Da) leaving a positive charge on the diacylglycerol. Both these MS routines allow the composition of phospholipids to be surveyed and together offer a powerful approach to targeted detection of oxidized phospholipids. The main fragmentations of value in targeted surveying of oxidized phospholipids are given in Table 1, although in negative-ion mode specific fragmentations for a number of individual fatty acid oxidation products can be used, as described previously [9,32,33].

These fragmentation routines can be carried out on any tandem mass spectrometer. However, it is important to note that several analyzer geometries exist, and some are limited in terms of the experiments that can be performed. Specifically, tandem instruments cannot perform additional fragmentations, denoted MS^3 or MS^n fragmentations (useful for enhanced structural identification of individual ions); this requires an ion trap instrument. On the other hand, ion traps cannot run precursor ion scanning, neutral loss scanning, or multiple reaction monitoring, as these all require linked scans in two separate analyzers, although more sophisticated instruments are capable of pseudo-routines that extract the necessary information from a full product ion mass spectrum. However, these routines may have significantly lower sensitivity as a result of the untargeted data collection.

Table 1
Specific ions and losses for oxidized phospholipids (only major diagnostic ions and losses shown).

	Positive ion	Negative ion
Headgroup		
Phosphatidylcholine	Precursor ion *m/z* 184	—
Phosphatidylserine	Neutral loss 185 Da	Neutral loss 87 Da
Phosphatidylethanolamine	Neutral loss 141 Da	—
Phosphatidylinositol	—	Precursor ion *m/z* 241
Sphingomyelin	Precursor ion *m/z* 184	
Oxidative modification		
Hydroperoxide (−OOH)	Neutral loss 34 Da	Neutral loss 34 Da
Chlorohydrin	Neutral loss 18/36/38 Da	Neutral loss 18/36/38 Da
Specific ox PL		
POVPC	Neutral loss 98 Da	Neutral loss 98 Da
PGPC	Neutral loss 114 Da	Neutral loss 114 Da
PONPC	Neutral loss 154 Da	Neutral loss 154 Da
PAzPC	Neutral loss 170 Da	Neutral loss 170 Da

Cardiolipin cannot be analyzed readily in a QTrap5500 because in the monovalent form its *m/z* is above the range of the instrument.

Materials

Materials

- Sep-Pak cartridges plus Ct18 (Product No. WAT 036810; Waters, Milford, MA, USA)
- Glass vials with screw cap (3.0 ml; Product No. 986297; Wheaton, Millville, NJ, USA)
- Crystal clear M/C Eppendorf tubes (0.6 and 1.5 ml; Product No. E1405; StarLabs, UK)
- Autosampler glass vials (0.3 ml) with screw top, fixed insert, clear with write-on patch (Product No. 500X03-FISV, C632; Chromacol, Spec & Burke Analytical)
- HPLC C8 Luna column (150 × 1.0 mm, 5 μm, 100 Å particle size; Product No. 00 F-4249-A0; Phenomenex, UK).
- Hamilton gastight syringes (50, 250, 500 μl; Fisher Scientific, UK)
- Glass measuring cylinders (carefully rinsed; see below)
- Amber glass vials (2.0 ml; Product No. 27083-U, Supelco, Bellefonte, PA, USA)
- Solid screw cap with PTFE liner for 2.0-ml vials (Product No. 27091-U; Supelco)
- Glass Pasteur pipettes

Chemicals

- Nitrogen (oxygen free), BOC (CAS 7727-37-9; Worsley, Manchester, UK)
- Argon (high purity), BOC (CAS 07440-37-1; Worsley)
- Sodium hypochlorite solution (Product No. 42,504-4; Sigma Aldrich Chemical Co., UK)
- Methanol (HPLC grade, Product No. M/4056/17; Fisher Scientific)
- Acetonitrile (HPLC grade, Product No. A/0627/17; Fisher Chemicals)
- Chloroform (HPLC grade, Product No. 650471; Sigma Aldrich Chemical Co.)
- Water (HPLC grade, Product No. 23595.328; VWR, UK)
- Formic acid (Optima, LC-MS grade, Product No. A117-50; Fisher Scientific)
- PAPC (Product No. 850459; Avanti Polar Lipids, USA)
- 1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (SOPC; Product No. 850467; Avanti Polar Lipids)
- 1-Stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (SAPC; Product No. 850469; Avanti Polar Lipids)
- POVPC (Product No. 870606; Avanti Polar Lipids)
- 1-Palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC; Product No. 870602; Avanti Polar Lipids)
- Phosphatidylethanolamines from sheep brain (Product No. P4264; Sigma Aldrich Chemical Co.)
- Dipalmitoylphosphatidylserine (DPPS; Product No. P1185; Sigma Aldrich Chemical Co.)
- Ammonium acetate (HPLC grade, Product No. A/3446/50; Fisher Scientific)
- Butylated hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol; Product No. 34750; Fluka, UK)
- *tert*-Butylhydroperoxide (Product No. B2633; Sigma Aldrich Chemical Co.)
- Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$; Product No. F7002; Sigma Aldrich Chemical Co.)
- Hanks' balanced salt solution (HBSS; Product No. H6136; Sigma Aldrich Chemical Co.)
- Carboxymethylimino-bis(ethylenenitrilo)tetraacetic acid (DPTA; Product No. D6518; Sigma Aldrich Chemical Co.)
- Ethylene glycol-bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA; Product No. E4378; Sigma Aldrich Chemical Co,)

Solvents and solutions

- HBSS: 1 HBSS tablet dissolved in 1 L of ultrahigh purity (UHP) water
- 100 mM NaOCl, pH adjusted to 6.0 by addition of small aliquots of HCl (**do not allow the pH to fall below 5.8,** otherwise chlorine gas will be released)
- Methanol/1% aq formic acid (90/10, v/v)
- Methanol/5 mM ammonium acetate (90/10, v/v)
- HPLC Solvent A: acetonitrile/5 mM ammonium acetate (10/90, v/v)
- HPLC Solvent B: acetonitrile/5 mM ammonium acetate (90/10, v/v)
- LC sample solvent: acetonitrile/5 mM ammonium acetate (50/50, v/v)
- Butylated hydroxytoluene: 0.05 mg/ml in methanol or chloroform

Equipment/instrumentation

- Vortex mixer
- Sonicating water bath
- Water bath
- Drying oven
- Microcentrifuge
- pH meter (Mettler Delta 320)
- QTrap 5500 (ABSciex, Warrington, UK) running Analyst software version 1.5.1
- Ultimate 3000 HPLC with autosampler (Dionex LC Packings, Dionex UK Ltd.) running Chromeleon XPress with DCMSLink

Protocol

Caution: handling of phospholipids in organic solvents

PLs dissolve readily in chloroform, but this solvent is also very efficient at extracting compounds, especially plasticizers and mold release agents from plasticware (microcentrifuge tubes, tips, etc.). Plasticizers such as polyethylene glycol give a strong set of repeating signals in the mass spectrum, easily spotted as these are separated by 44 Da (corresponding to one of the monomer units that make up the polymer) and tend to have a Gaussian distribution. Polypropylene glycol (repeating unit 68) is also a common contaminant, and many detergents contain these compounds. Therefore chloroform solutions should be used only in glass vials and dispensed using glass syringes with stainless steel needles. Wherever feasible, avoid the use of plastic tubes and pipette tips, especially cheap ones, and the use of detergents in washing glassware. The use of glassware for storage of solvents and samples comes also with a price, as often phospholipid sodium adducts ($[MNa]^+$) are observed in the MS spectrum even if no sodium salts were used during preparation of the HPLC buffer. The contribution of proton ($[MH]^+$) and sodium adducts should be considered when interpreting phospholipid mass spectra. Chloroform is also very volatile and evaporates rapidly. Sample vials containing phospholipids dissolved in chloroform should be left uncapped as little as possible, and care needs to be exercised in assuming concentrations remain constant after repeated handling. We aliquot (0.1–0.5 mg) all phospholipids that are supplied as chloroform solutions immediately on opening and store them dried under inert gas at −80 °C.

Caution: solvent hazards

All organic solvents are hazardous, and volumes over 10 ml should be handled in a fume cupboard and with appropriate safety measures. Chloroform is a suspected carcinogen and methanol is toxic.

Preparation of oxidized phospholipids in vitro

The following method is for preparation of autoxidized PAPC or any other unsaturated phospholipid.

(1) Place 100 μl of 1 mg/ml PL solution in methanol in a clean glass vial (no cap). Dry the PL solution onto the sides of the vial as a fine layer under a stream of oxygen-free nitrogen.
(2) Wrap the tube in foil (to limit photolytic reactions) and leave the vial open at room temperature for 5 days to autoxidize.
(3) Add 100 μl of methanol and vortex.
(4) Remove 1 μl and dilute with 999 μl of methanol–formic acid in a glass vial (avoid rubber caps and polyethylene-based plastics).
(5) Check the autoxidation status by direct infusion as described below.

Preparation of phospholipid chlorohydrins in vitro

First prepare phospholipid vesicles in an aqueous environment and oxidize them with NaOCl, as follows.

(1) Place a 40-μl aliquot of 10 mg/ml phospholipid solution in methanol into a 1.5-ml Eppendorf tube.
(2) Dry under nitrogen.
(3) Add 40 μl of HBSS.
(4) Vortex for 1 min.
(5) Sonicate in a water bath at 37 °C for 15 min.
(6) Vortex again to give a milky, homogeneous suspension (this can be stored at −20 °C or used immediately).
(7) Add 50 μl of 50 mM NaOCl solution, pH ~6.0.
(8) Add 110 μl of HBSS and vortex.
(9) Incubate for 30 min at 37 °C.

The reaction is terminated and excess HOCl is removed using a reverse-phase Sep-Pak cartridge.

(1) Prime the Sep-Pak cartridge by passing through 2 ml of methanol (apply using a syringe).
(2) Equilibrate the cartridge with at least 2 ml of UHP water.
(3) Load the chlorohydrin preparation, wash the tube with 0.3 ml of water, and load this, too, discarding the eluent from the cartridge.
(4) Wash with a further 1 ml of UHP water to remove excess salts.
(5) Apply 0.5 ml of methanol, discarding the first 0.1 ml of eluent (still aqueous), but then collecting all subsequent eluent in a clean glass vial.
(6) Apply 1 ml of methanol:chloroform (1:1, v/v) to elute all the phospholipids.
(7) Dry the 1.4 ml of eluent in organic solvent under nitrogen gas. The phospholipid chlorohydrin preparation can be stored frozen at < −20 °C for 1–2 weeks.

Extraction of phospholipids from cells, plasma, or LDL

Phospholipids can be extracted from a wide variety of biological materials using similar protocols. Extraction of tissue samples usually involves freeze-clamping and grinding in liquid nitrogen before extraction with organic solvents [9], whereas cell samples can be extracted directly after washing to remove culture or preparation medium [34]. Liquid samples such as plasma or serum can likewise be extracted without freezing. However, in all cases it is essential to minimize oxidation during sample processing: an antioxidant (e.g., butylated hydroxytoluene) and a metal chelator (e.g., DPTA or EGTA) should be added to give a final concentration of approximately 100 μM. Reducing agents such as $SnCl_2$ or borohydride can also be included [9]; these stabilize hydroperoxides by converting them to hydroxides, but this means that the hydroperoxides themselves cannot be measured. We have found the method of Folch et al. [35] to be best for extracting phospholipids from LDL, and this method is given below.

(1) Place 40 μl of desalted LDL (corresponding to ~25 μg of protein) in a 1.5-ml Eppendorf tube (MS grade).
(2) Add 160 μl of ice-cold methanol and vortex for 30 s; then add 100 μl of ice-cold chloroform containing 50 μg/ml BHT and vortex again. Add a further 240 μl of chloroform, vortex for 1 min, and allow the mixture to equilibrate for 20 min on ice with occasional vortex mixing.
(3) Add 150 μl of UHP water to the mixture, vortex for 1 min, and keep on ice for an additional 10 min with occasional vortex mixing. After addition of the water, two immiscible layers should be clearly visible.
(4) Centrifuge the mixture for 5 min at 5000 *g* in a microcentrifuge to separate the aqueous and organic phases.
(5) Remove upper layer (aqueous) using a clean glass Pasteur pipette into a new Eppendorf tube, taking care not to leave any aqueous phase. It does not matter if a small volume of organic phase is also removed. Retain the (lower) organic layer.
(6) Perform a second extraction step of the aqueous phase by the addition of 250 μl of ice-cold chloroform:methanol (2:1, v/v) to increase recovery of lipids from samples, and leave on ice with occasional vortex mixing for 10 min.
(7) Centrifuge for 5 min at 5000 rpm; remove all the upper (aqueous) phase and discard.
(8) Combine both organic phases in a single microcentrifuge tube, and wash with 200 μl of UHP water, and vortex. Centrifuge, and carefully remove and discard upper (aqueous) phase.
(9) Dry the chloroform phase containing the lipids under a stream of nitrogen (oxygen free) and store at −80 °C.

Note. For longer term storage (more than 1 week) it is better to store samples under argon as an inert gas, as it is heavier and disperses less easily.

Preparation of samples for MS analysis

Individual commercial lipid standards should be run at 10–100 ng/ml for phosphocholine-containing lipids and 0.1–1 μg/ml for other phospholipids. Phospholipid mixtures can be used at approximately 0.1–1 μg/ml. For cells or tissue, extract 10–50 mg of tissue, and after drying reconstitute in 100 μl of methanol:chloroform (4:1; v/v), and make 1/10 and 1/100 dilutions for testing (test the lower concentration first).

Caution: solvent preparation

Many detergents contain polyethylene or polypropylene glycols, which give strong MS signals. Glassware for preparation of the HPLC and MS solvents must not be washed in detergent. Before the first use, rinse as follows: under running tap water for 5 min, five times with UHP water, three times with small volumes of methanol. Dry in a drying oven. Keep glassware for solvents separate and capped with tin foil, and subsequently rinse only with methanol or UHP water.

MS analysis—general considerations

The instrument should have been recently calibrated using the calibrant solutions (polyethylene glycol, polypropylene glycol, peptide/-protein, or other standard solutions according to the manufacturer's guidelines) provided by ABSciex and according to the manufacturer's guidelines. Use a separate syringe and tubing for infusing calibrant solutions as these compounds are persistent and give strong signals even at low concentrations. The source tuning parameters should always be checked using infusion of solutions of the

respective phospholipids in positive or negative mode, as appropriate (0.1–1 μg/ml for phosphatidylcholines (PCs) and 10-fold higher concentrations for other phospholipids).

Typical parameters for analysis of phospholipids in several different MS routines are given in Table 2. However, as every instrument is set up slightly differently, we recommend checking the parameters for the advanced routines using solutions of oxidized PLs prepared in vitro, before running experiments to analyze biological samples. This can be done by direct infusion of the sample; protocols are given below. Masses for selection in scanning or fragmentation modes should be entered with accuracy to one decimal place for the exact monoisotopic mass of the molecular ion.

Before starting the sample analysis, infuse 200 μl of solvent to check that the detector is operating (solvent cluster peaks will be observed at low *m/z*) and that there are no significant contaminants, for example, left over from previous samples.

Critical

It is important to wash the direct infusion syringe extensively between samples to limit the occurrence of cross-contamination. The Peek tubing connecting the syringe to the source should likewise be flushed through with the loading or running solvent. The same applies to the Hamilton syringes used to prepare dilutions of standards or samples. The use of chloroform or dichloromethane is not recommended for routine washing, as long-term use may damage many components of the HPLC system, although chloroform:methanol (1:1, v/v) can be used occasionally to deal with serious contamination issues.

Recording a simple spectrum of the PL sample

(1) Using Hamilton syringes, dilute the sample of oxidized PL to 1 μg/ml in 90% methanol–10% 5 mM ammonium acetate.
(2) Take up 200 μl into the delivery syringe, taking care to remove bubbles.
(3) Connect the syringe to the source and insert into the syringe pump.
(4) Infuse the sample initially at 10–15 μl/min until the signal is observed (e.g., as seen in Figs. 2 or 4A), then reduce the flow to 2–3 μl/min.
(5) In the MS menu, select "EMS" (enhanced mass scanning) and check that the parameters are set up according to Table 2 or a similar optimized parameter set. This can be carried out in positive- or negative-ion mode, but note that the parameter settings are different for these two modes.
(6) Check that "dynamic fill" is selected in the Advanced MS menu.
(7) Start scanning. If the signal is satisfactory, i.e., peaks of expected *m/z* can be seen (Tables 3 and 4), acquire data for 1–2 min (data are saved to disk).

Checking fragmentation patterns of specific PLs

If individual oxidized phospholipids are of particular interest, peaks at the relevant *m/z* can be fragmented to confirm their identity.

(1) Set the mass range from 100 *m/z* to just above the molecular ion chosen for fragmentation.
(2) Select "EPI" (enhanced product ion) in the MS menu.
(3) Enter the *m/z* of the molecular ion to be fragmented (e.g., *m/z* 790.6, 818.6, 846.6, etc.).
(4) Start scanning with collision energy 10 eV, and then increase it until adequate fragmentation is observed. Note that some product ions occur only at certain collision energies.
(5) Use these parameters to acquire data.
(6) If information about the fragmentation of product ions is required, select "MS3" in the MS menu and enter the *m/z* of the first and second precursors. Set the collision energy to the optimum observed in step (4).
(7) Start scanning, and increase the excitation energy until the optimum fragmentation is achieved (range 0–1).

Targeted scanning for phosphocholines and phosphoethanolamines

To select the phosphocholine-containing phospholipids (phosphatidylcholine and sphingomyelin), precursors of *m/z* 184.1 can be used. Most of the parameters should remain the same as for EMS unless specifically mentioned; some are changed automatically.

(1) Set the polarity to positive-ion mode.
(2) Select "precursor ion scanning" in the MS menu and enter 184.1 for the precursor.
(3) Scan, and during this check various settings of collision energy (30–50 eV) to obtain the most productive fragmentation, as

Table 2
Typical parameter settings for the QTrap5500 in different scan modes.

Parameter	Positive-ion EMS	Negative-ion EMS	LC–MS (positive ion)	Precursor ion (positive ion)	Neutral loss (positive ion)
MS					
Mass range (*m/z*)	400–1000	400–1000	400–1000	400–1000	~600–1000
Scan rate (Da/s)	10,000	10,000	[a]	1000	200
Source/gas					
Curtain gas	20	20	25–30	20	20
Collision gas	High	High	High	High	High
Ion spray voltage (V)	5,500	−4,500	5500	5500	5500
Temperature (°C)	50	50	100–120	50	50
Compound					
Declustering potential	70–100	70–100	70–100	70–100	70–100
Collision energy (eV)	10	10	[a]	30–50	30–50
Resolution					
Q1	n/a	n/a	[a]	Unit	Low
Q1 ion energy	1.0	−1.0	[a]	0.8	n/a
Q3	n/a	n/a	[a]	Unit	Unit
Q3 ion energy	n/a	n/a	[a]	1.0–1.4	1.0–1.3
Advanced MS					
Scan mode	Profile	Profile	Profile	Profile	Profile
Step size	0.1	0.1	[a]	0.12	0.2–0.5
Dynamic fill time	Yes	Yes	Yes	Yes	Yes

[a]Depends on the MS routine(s) used. When interfacing with LC, some of the parameters for the scanning modes need to be changed because of the higher flow rates used (e.g., temperature and gas flow rates).

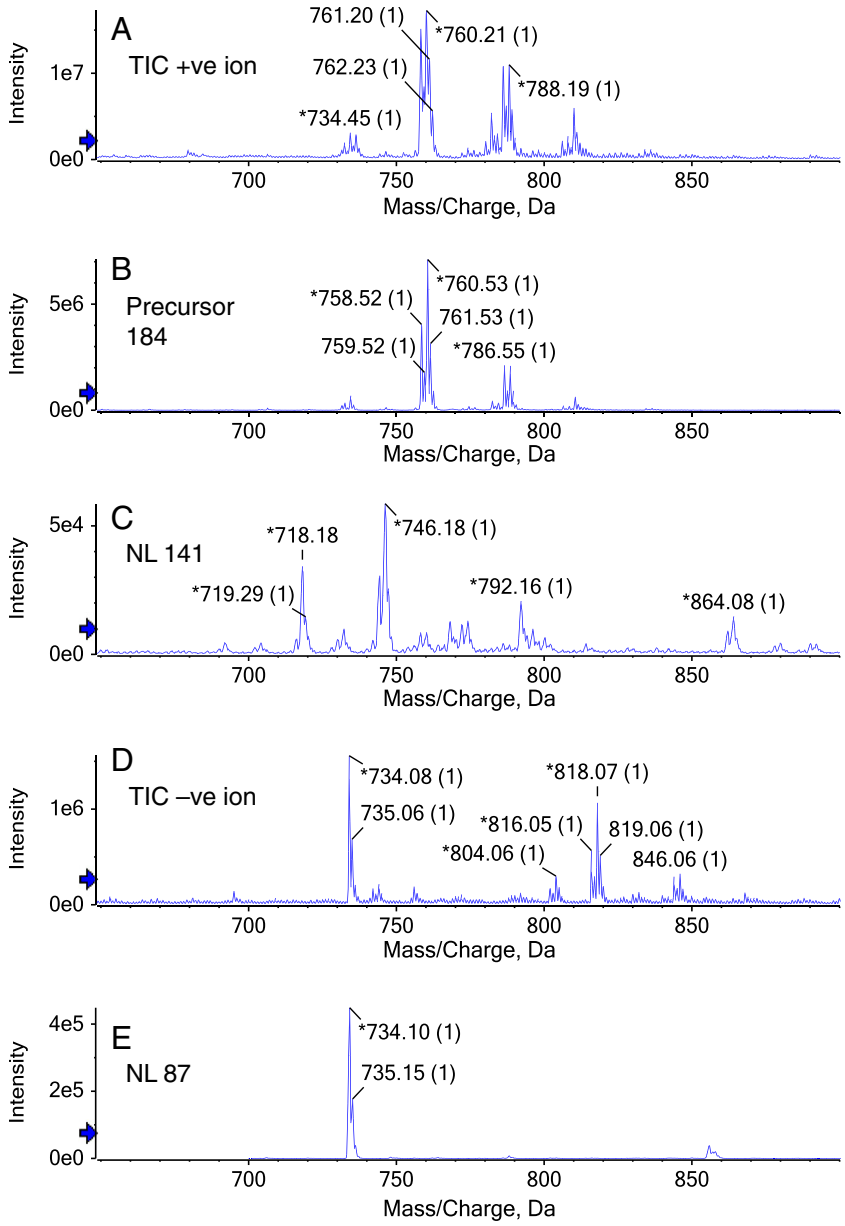

Fig. 4. Selection of phospholipid types by headgroup-specific scanning routines. An equimolar mixture of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) was infused directly at a flow rate of 3 μl/min. (A) EMS spectrum in positive-ion mode, showing PCs with strongest signals. (B) Scanning for precursors of 184 *m/z* in positive-ion mode, selective for PCs. (C) Scanning for neutral loss of 141 Da in positive-ion mode, selective for PEs. (D) EMS spectrum in negative-ion mode, showing DPPS with the strongest signal and PCs observed as the acetate adducts (+58 cf. (B)). (E) Scanning for neutral loss of 87 Da in negative-ion mode, selective for PS and showing DPPS at *m/z* 734.1.

indicated by the maximum absolute intensity of the observed signal. (*Note.* Excessive collision energies can result in reduced signal intensities.)

(4) Acquire the data using the optimum parameters established in step (3).
(5) Select "neutral loss" in the MS menu and enter 141.1 (detects phosphoethanolamines).
(6) Start scanning; if signal intensity is too weak, change resolution in Q1 to low.
(7) Check the optimum collision energy as described for PC above, then acquire data.

Note

Settings for precursor ion and neutral loss scanning can be adjusted to optimize for different requirements, for example, by adjusting what is referred to as "dwell time," which is the time taken collecting data at each step during scanning in Q1. This is defined by the mass range over which the scan is performed, the time taken for this scan, and the "step size" in Q1. The dwell time is given by the scan time divided by the mass range multiplied by the step size. A dwell time of 10–50 ms is usually sufficient. Longer dwell times give better sensitivity (signal to noise), but at a cost of either lower resolution in the mass spectrum or fewer data points per unit time, which makes quantification from liquid chromatography less reliable. For statistical purposes, at least 7 to 9 points across a chromatography peak are required. There is no hard and fast rule as to what is best, as it depends on the sample. Resolution settings also affect sensitivity, with lower resolution settings giving better sensitivity at the cost of selectivity. For increased sensitivity it is usually best to reduce resolution in Q1 first, as maintaining selectivity for the reporter fragment is usually more beneficial. Optimization of the collision energy is also important

Table 3
Mass-to-charge ratios of some commonly observed phospholipids.

Positive-ion mode							
PC[a,b] H (Na)	FA[c]	SM H (Na)	FA	PE	FA	O-PE[d]	FA
732 (754)	16:0/16:1	703 (725)	16:0	716	16:0/18:2	724	16:0/20:4
734 (756)	16:0/16:0	731 (753)	18:0	740	16:0/20:4	750	18:1/20:4
							18:0/20:5
758 (780)	16:0/18:2	787 (809)	22:0	742	18:0/18:3	752	18:0/20:4
760 (782)	16:0/18:1	813 (835)	24:1	744	18:0/18:2	748	16:0/22:6
782 (804)	16:0/20:4	843 (865)	26:0	764	16:0/22:6	776	18:0/22:6
					18:2/20:4		
784 (806)	18:0/18:3			768	18:0/20:4		
786 (808)	18:0/18:2			792	18:0/22:6		
806 (828)	16:0/22:6			794	18:0/22:5		
810 (832)	18:0/20:4						

Negative-ion mode					
PS[e]	FA	PI	FA	PE	FA
734	16:0/16:0	807	16:0/16:1	714	16:0/18:2
758	16:0/18:2	833	16:0/18:2	738	16:0/ 20:4
760	16:0/18:1	835	16:0/18:1	740	18:0/ 18:3
786	18:0/18:2	861	18:0/18:2	742	18:0/ 18:2
788	18:0/18:1	885	18:0/20:4	762	16:0/ 22:6
810	18:0/20:4			766	18:0/ 20:4
812	18:0/18:3			790	18:0/ 22:6

PC, phosphatidylcholine; FA, fatty acid; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

[a] Phosphocholine-containing phospholipids can exist as the Na adduct (+22 compared to protonated form). K adducts (+38) are also possible.

[b] PCs can be observed as acetate adducts in negative-ion mode, if samples are analyzed in the presence of ammonium acetate. The *m/z* is 58 Da higher than $[M]^+$.

[c] The likely fatty acyl chain composition, but isobaric forms also exist.

[d] Plasmenyl phospholipids have a vinyl ether linkage instead of an ester bond, with *m/z* 16 less than the corresponding ester. They are common for PE and also PC (not listed).

[e] PS can be observed also in positive-ion mode but with weak intensity; the *m/z* is 2 higher than in negative-ion mode.

to achieve the best sensitivity during survey scans, as the amount of energy required varies between different phospholipids and phospholipid oxidation products (phospholipid chlorohydrins, phospholipid hydroperoxides, and chain-shortened phospholipids). On our system settings of 40–45 eV were optimal, and longer chain phospholipids needed slightly higher energy.

Targeted scanning for phosphoserines

(1) Set the instrument to negative ion mode.
(2) Select "neutral loss" in the MS menu and enter 87.0 for the loss (selects phosphatidylserines). Check the optimum collision energy and resolution as described above, then acquire data.

Targeted detection of hydroperoxides and chlorohydrins

Hydroperoxides and chlorohydrins or other chlorinated species can be targeted by looking for neutral losses of 34 Da (H_2O_2) and 36/38 Da ($H^{35}Cl/H^{37}Cl$); this can be done in either positive- or negative-ion mode depending on the species of phospholipid of interest. The parameters below are for phosphatidylcholines, as an example. Ions containing –OH groups (including chlorohydrins and hydroperoxides) also lose H_2O (−18 Da).

(1) Set the instrument to the desired ion mode depending on the type of PL to be analyzed (positive ion for PC or PE; negative ion for PS).
(2) Select "neutral loss" in the MS menu and enter 34.0 for the loss (selects hydroperoxides).
(3) Set the resolution in Q1 to low and in Q3 to unit (ion energy 1.0).
(4) Set the collision energy to 45–50 eV.
(5) Start scanning to check the parameters, then acquire data.
(6) Repeat steps (2)–(5) for neutral losses of 36.0 and 38.0 Da.
(7) For neutral loss of 18.0 Da, reduce the collision energy to ~30 eV. Scan and acquire data as above.

Detection of individual chain-shortened PLs

Phospholipids containing specific oxidized moieties, such as oxovaleroyl or glutaroyl chains in the *sn*-2 position, can also be detected using neutral loss scanning. The most common forms are derived from PAPC but stearoyl arachidonoyl PC can also yield these oxidations.

(1) Set the instrument to the desired ion mode depending on the type of PL to be analyzed.
(2) Select "neutral loss" in the MS menu and enter 98.0 for the oxovaleroyl (C5 aldehyde), 114.0 for glutaroyl (C5 carboxylic

Table 4
Mass-to-charge ratios of some typically observed oxidation products of PC and PE.

m/z ratio ($[MH]^+$)	PC molecular species	OOH	$(OOH)_2$	HOCl
(a) Oxidation and chlorination products of phosphatidylcholines				
758.6	16:0/18:2	790.6	822.6	810.6
760.6	16:0/18:1	792.6	–	812.6
782.6	16:0/20:4	814.6	846.6	834.6
786.6	18:0/18:2	818.6	850.6	838.6
788.6	18:0/18:1	820.6	–	840.6
806.6	16:0/22:6	838.6	870.6	858.6
810.6	18:0/20:4	842.6	874.6	862.6
m/z ratio ($[M+H]^+$)	PE molecular species	OOH	$(OOH)_2$	HOCl
(b) Oxidation and chlorination products of phosphatidylethanolamines				
724.6	16:0/20:4	756.5	788.5	776.5
750.6	18:1/20:4	782.5	814.5	802.5
752.6	18:0/20:4	784.5	816.5	804.5
764.6	18:2/20:4	796.5	828.5	816.5
768.6	18:0/22:6	800.5	800.5	820.5

Isotope	ClOH	$(ClOH)_2-H_2O$	$(ClOH)_2$	$(ClOH)_3-H_2O$	$(ClOH)_3$	$(ClOH)_4-H_2O$	$(ClOH)_4$
(c) Series of isotope peaks for chlorohydrins for PAPC (m/z 782.6)							
^{35}Cl	834	868	886	920	938	972	990
$^{37}Cl\times1$	836	870	888	922	940	974	992
$^{37}Cl\times2$	–	872	890	924	942	976	994
$^{37}Cl\times3$	–	–	–	926	944	978	996
$^{37}Cl\times4$	–	–	–	–	–	980	998

Chlorohydrins can also lose HCl (−36 Da) to give intermediate series of signals, e.g., PAPC $(ClOH)_4-HCl$ gives the series 754/756/758, etc., indicated in Figs. 8D and E.

acid), 154.1 for 9-oxononanoyl (C9 carboxylic acid), or 170 for azelaoyl (C9 carboxylic acid) moiety.
(3) Set the resolution in Q1 to low and in Q3 to unit (ion energy 1.0).
(4) Set the collision energy to 45–50 eV.
(5) Start scanning to check the parameters; then acquire data.

Once the optimal parameters for known oxidized phospholipids have been determined for the available mass spectrometer, the procedures can be extended to liquid chromatography and analysis of biological or clinical samples.

LC–MS analysis of oxPL

Liquid chromatography of phospholipids and oxidized phospholipids has been reported using a wide variety of columns and stationary phases. Whereas normal-phase chromatography is often used for separating different classes of phospholipid, reverse phase works better for separating oxidized from native phospholipids. Phospholipids with long fatty acyl chains are quite nonpolar, so highly organic solvent systems are needed to remove them from C18 columns, whereas with C4 columns the more polar oxidized and chain-shortened forms elute at the solvent front unless solvents with high aqueous content are used (in which other phospholipids may not be soluble). Hence a C8 stationary phase offers the best flexibility for overall separation.

(1) Connect the column inline between the LC system and the source, ensuring no leaks.
(2) Set the flow rate to 100 μl/min and equilibrate with Solvent A for at least 15 min.
(3) Scan briefly to ensure that the instrument is responding.
(4) In Chromeleon (or the software for an alternative LC system), set up the chromatography gradient program as follows:
(a) 0 to 3 min, 50% Solvent B;
(b) 3 to 10 min, gradient to 100% Solvent B;
(c) 10 to 33 min, 100% Solvent B;
(d) 33 to 35 min, gradient to 50% Solvent B;
(e) 35 to 40 min, 50% Solvent B.
(5) In Analyst (or the software for an alternative MS system), set up the methods for scanning during the chromatography run. Ensure that the length of the scanning program matches the length of the chromatography program. Different methods can be set up, depending on the phospholipid type and oxidation product of interest. For example, for phosphatidylcholines:
(a) EMS, precursor of 184.1, neutral loss of 34 (detection of PC hydroperoxides);
(b) EMS, neutral loss of 36, neutral loss of 38 (detection of PC chlorohydrins).

Note that there is a limit to the number of MS routines that can be incorporated into one chromatography run. The scan rates for precursor ions and neutral losses are necessarily slower than for simple mass scanning (200–1000 as opposed to 10,000 Da/s), as including several scanning experiments in the same routine lengthens the duty cycle of the MS. If the duty cycle is too long, the chromatographic data will appear quantized; therefore the duty cycle should not be longer than approximately 5 s (for good quality quantification using the area under the chromatographic peak, as 9–15 data points are needed across the chromatographic peak). To scan for a wide range of oxidized or chlorinated phospholipids, it may be necessary to carry out more than one chromatographic run.

Sample batch preparation for LC–MS

Samples are prepared in acetonitrile:5 mM ammonium acetate (50:50, v/v). In the batch, include a sample of oxidized phospholipids prepared in vitro and characterized. It is also essential to include blank runs to assess background signal and carryover between runs; these involve injection of acetonitrile:5 mM ammonium acetate (50:50, v/v) and are usually done at the beginning and end of the batch. However, for batches of more than about 10 samples, additional blank runs should be incorporated between samples.

(1) Set up the queue for the samples to be run, specifying the MS and LC programs to be run for each sample. **Critical:** check that the run durations of both programs are the same.
(2) The injection volume should be 10 or 20 μl. Load the samples into glass autosampler vials with inserts. The amount to be loaded will depend on the nature of the autosampler needle and the settings employed, but usually a small excess over the injected volume is required.
(3) Ensure that there is sufficient solvent in the reservoirs to last for the duration of the run.
(4) Start the batch; check that the LC and MS are communicating correctly and data are being acquired.

Critical

To prevent carryover and cross-contamination of samples, extensive washing of the injection system is required between samples. The needle should be washed with methanol (rather than the running solvent) for best solubilization and removal of lipids, and a total wash program of at least 1 ml is recommended.

Sensitivity and limits of detection

On the QTrap5500, the limit of detection for a single phosphocholine species is approximately 0.125 pmol (100 pg) using EMS and neutral loss scanning, with a further gain in sensitivity of at least 10-fold using precursor ion scanning, owing to the improvement in signal-to-noise ratio. This is based on an injection volume of 10 μl. The limit of detection for other phospholipids is lower, as the ionization efficiency is not as good. However, the sensitivity of different MS instruments varies, so the limit of detection should be checked. Using the targeted survey experiments, oxidized species can be identified at levels of ~0.1% of the unmodified lipids.

Data analysis

Data files (.wiff) can be analyzed either using the Explorer function of Analyst or in Peakview. For direct infusion data, the ion intensity can be used as an approximate indicator of concentration when referenced to an internal standard. For chromatographic data, the peaks can be integrated and compared to the standard. To identify the presence of hydroperoxides, precursor ion scanning for the phospholipid type should be correlated with the neutral loss of 34 Da. When looking for chlorinated species, there should be neutral losses of both 36 and 38 Da, as loss of 36 Da alone may also arise from loss of two water molecules. Experiments using precursor ion and neutral loss scanning are in practicality less quantitatively accurate than multiple-reaction monitoring, and the best application of this approach is for comparative analyses between controlled samples, rather than absolute quantitation.

At the simplest level, the *m/z* value can be used to predict the total number of carbons and degree of unsaturation of the fatty acid chains and therefore narrow down the identity of phospholipids. For the comprehensive analysis of lipids at the cellular and physiological level, a more accurate knowledge of the phospholipid structure is required. Currently, a number of Web services and software programs designed to assist scientists undertaking research in the field of lipidomics are available. These include public lipid databases with associated search engines (Cyberlipids, LipidMaps, LipidNavigator, and others) containing structures of phospholipids, including sphingolipids, and algorithm-based programs (such as LIMSA, LipidProfiler, LipidView, LipidQA, LipidInspector, LipID, Lipid Data Analyzer) for the automated processing of MS data. The database search engines usually allow the researcher to

input the molecular weight of the lipid and to retrieve potential molecular formulae and in some cases structures. The searches can be further modified and refined based on prior knowledge using predetermined settings (such as lipid class, mass error, etc.). Each database interface has critical issues to bear in mind; for example, with the LipidMaps database, PC identification needs the *m/z* value for the zwitterionic form to be entered, whereas others require the protonated *m/z*. The algorithm-based software programs either have internal databases or can be interfaced with the public databases available on the Web. They usually take mass spectrometry data (generally in a portable format such as mzXML, although some will take raw data files) and match the data to databases, outputting a list of identified formulae and, in a few cases, identification based on MS^2 data. Some are specific and limited to the processing of high-resolution, high-mass-accuracy data obtained with FT or Orbitrap instruments (e.g., LipID), and others do not support data-dependent acquisition methods (e.g., LIMSA) or do not offer correction for overlapping peaks due to the isotopic distribution. An advantage of some of the available software packages is that they allow 2D chromatographic visualization that can be used to screen the elution profiles of samples and identify outlier runs when analyzing a large set of samples before automated processing. It is worth noting that most of these tools were initially developed for the field of metabolomics and have been adapted to lipidomics with new databases. Most of the databases include native lipids only and are not set up for the analysis of oxidative modifications, although in theory oxidized lipids could be added to the databases. Even so they can be useful for identifying which peaks may correspond to known, unmodified lipids, allowing more focused analysis of potentially modified ones. Choosing the best tool will depend on the instrument and application. For a more comprehensive account of the challenges of translating raw data into meaningful lipid changes at the physiological level, some recently published reviews are recommended [36–39].

Calculations and expected results

Standard mass spectrometry allows the observation of a variety of oxidized phospholipids within a mixture; polyunsaturated phospholipids such as PAPC can yield an extensive family of oxidation products, as shown in Fig. 2. The peroxidation process involves the addition of multiples of molecular oxygen (O_2; +32 Da), but hydroperoxides can undergo rearrangement and loss of water (−18 Da), as well as fragmentation to chain-shortened or lysolipids. The *m/z* ratios of some common phospholipids and their oxidation products are given in Table 3. In positive-ion mode, by far the strongest signals are from PCs, as these have a constitutive positive charge; PEs and PSs give minor contributions to the spectrum, even if present in equimolar amounts, as illustrated in Fig. 4. Scanning for precursors of 184.1 *m/z* identifies the PCs (Fig. 4B), and neutral loss of 141.1 Da identifies the PEs (Fig. 4C); the ion current for the latter is approximately 100-fold lower than for the PCs. On the other hand, this demonstrates the potential of survey scanning routines to identify minor components of the spectrum with high signal to noise. In negative-ion mode, PS species can be observed, together with signal from the acetate adducts of PCs (+58 compared to the *m/z* in positive mode) and weak contributions from PEs (Fig. 4D). PS species can be identified by neutral loss of 87 Da in negative-ion mode; Fig. 4E shows how specific this method is, with the only signal coming from dipalmitoyl PS at 734 *m/z*. In general, these methods enhance the signal-to-noise ratio and offer great improvements in specificity for complex samples or ones containing contaminants.

Neutral loss of 34 Da is a targeted approach for identifying hydroperoxides of phospholipids and has been described previously [22]. The levels of oxidized phospholipids are often very low in biological samples and, in a normal MS spectrum, may be masked by the presence of native PLs, as in Fig. 5A, but the neutral loss scanning (Fig. 5B) allows their detection often even at low levels. Figs. 5C and D show that the neutral loss of 34 Da is quite specific; neutral loss of 36 and 38 Da gives very little signal (the small peaks visible at different masses are from potassium adducts). This is important, as neutral loss of 36 and 38 Da can be used for identification of chlorine-containing phospholipids (Figs. 5E–H). In this example, SOPC monochlorohydrin at 840 *m/z* shows loss of 36 ($H^{35}Cl$) and 38 ($H^{37}Cl$) Da, but no loss of 34 Da. The signals at 822 *m/z* correspond to a dehydrated form; chlorohydrins also show loss of −18 Da. The combination of headgroup identification and scans of oxidative modifications provides confidence in detecting oxidized phospholipids.

Lipid peroxidation generates chain-shortened species containing aldehyde and carboxylic acid termini. These specific moieties can be targeted by using neutral loss of the mass of the oxidized chain, such as loss of 98 Da for POVPC and 114 Da for PGPC (Fig. 6); this identifies any lipids containing the oxovaleroyl and glutaroyl chains. In Fig. 6, the neutral loss of 114 Da demonstrates the presence of both the protonated and the sodiated forms of PGPC at 610 and 632 *m/z*, respectively, although there are very minor signals from longer chain oxidized phospholipids that can fragment to yield these products. Typically there is also a small signal at −18 *m/z* for both PGPC and POVPC, corresponding to their dehydration products. Analogous results would be obtained for SOVPC and SGPC. Similar approaches can be used for other chain-shortened phospholipids (Table 1). However, as the neutral loss is essentially selective for a small number of species, there is an argument for using MRM to select an individual ion of interest, as MRM does not scan in Q1 and therefore has a much shorter duty cycle. Note that for phospholipids that can be observed best in negative-ion mode, this experiment can be conducted either as a neutral loss for the masses given in Table 1 or as a precursor ion scan for the negatively charged moieties at $[M-H]^-$ (mass minus 1 Da).

The structure of a particular molecular ion appearing in the spectrum of a mixture of phospholipids can be checked to determine whether it is oxidized. Fig. 7 shows the expected fragmentation patterns for some hydroperoxide-containing phospholipids. The strong signal at 184 *m/z* identifies the lipids as those with phosphocholine-containing headgroups, and the fragmentations of PLPC monohydroperoxide at 790 *m/z* and SAPC bis-hydroperoxide at 874 *m/z* show characteristic losses of 18 and 34 Da. In contrast, the chlorohydrin of SOPC at 840 *m/z* shows a typical loss of 18 and 36 Da. The QTrap is capable of carrying out MS^3 fragmentation, which can be useful for more detailed investigation of structures, for example, showing in Figs. 7D and E that the oxidized PAPC species 846 *m/z* fragments differently to the other hydroperoxides and in fact has rearranged to an endoperoxide. The EPI MS^2 fragmentation shows loss of 18 and 36 Da, which could be confused with a chlorohydrin, but MS^3 fragmentation of the 828 *m/z* product ion shows that the ion at 810 *m/z* is formed by a second loss of water. Scanning for neutral loss of 36 and 38 Da is able to discriminate between chlorohydrins and endoperoxides.

For complex mixtures of phospholipids, such as those deriving from biological or clinical samples, the best approach is to separate oxidized from native phospholipids by reverse-phase liquid chromatography. Using a C8 column with the gradient program given in the protocol, the phospholipid separation expected is shown in Fig. 8. Essentially, all lysolipids and oxidized and chain-shortened phospholipids elute in the first 20 min, whereas unoxidized lipids elute from approximately 20 min onward. In this experiment, precursors of 184.1 *m/z* were used to identify phosphocholine-containing species, neutral loss of 34 Da identified hydroperoxides, and loss of 36/38 Da identified chlorohydrins. Comparison with the EMS analysis (Fig. 8A) shows that many of the species identified by targeted scanning were minor components of the phospholipid mixture. The first phospholipid species to elute are lysolipids and chain-shortened oxidized lipids (Fig. 8B): the chromatogram contains many different oxidized species, but the lysopalmitoyl PC (496 *m/z*), lysostearoyl PC (524 *m/z*), PGPC (610 *m/z*), and POVPC (594 *m/z*) have been indicated. Fig. 8C shows the detection of several hydroperoxides by neutral loss of 34 Da: the strongest signal was from PLPC hydroperoxide

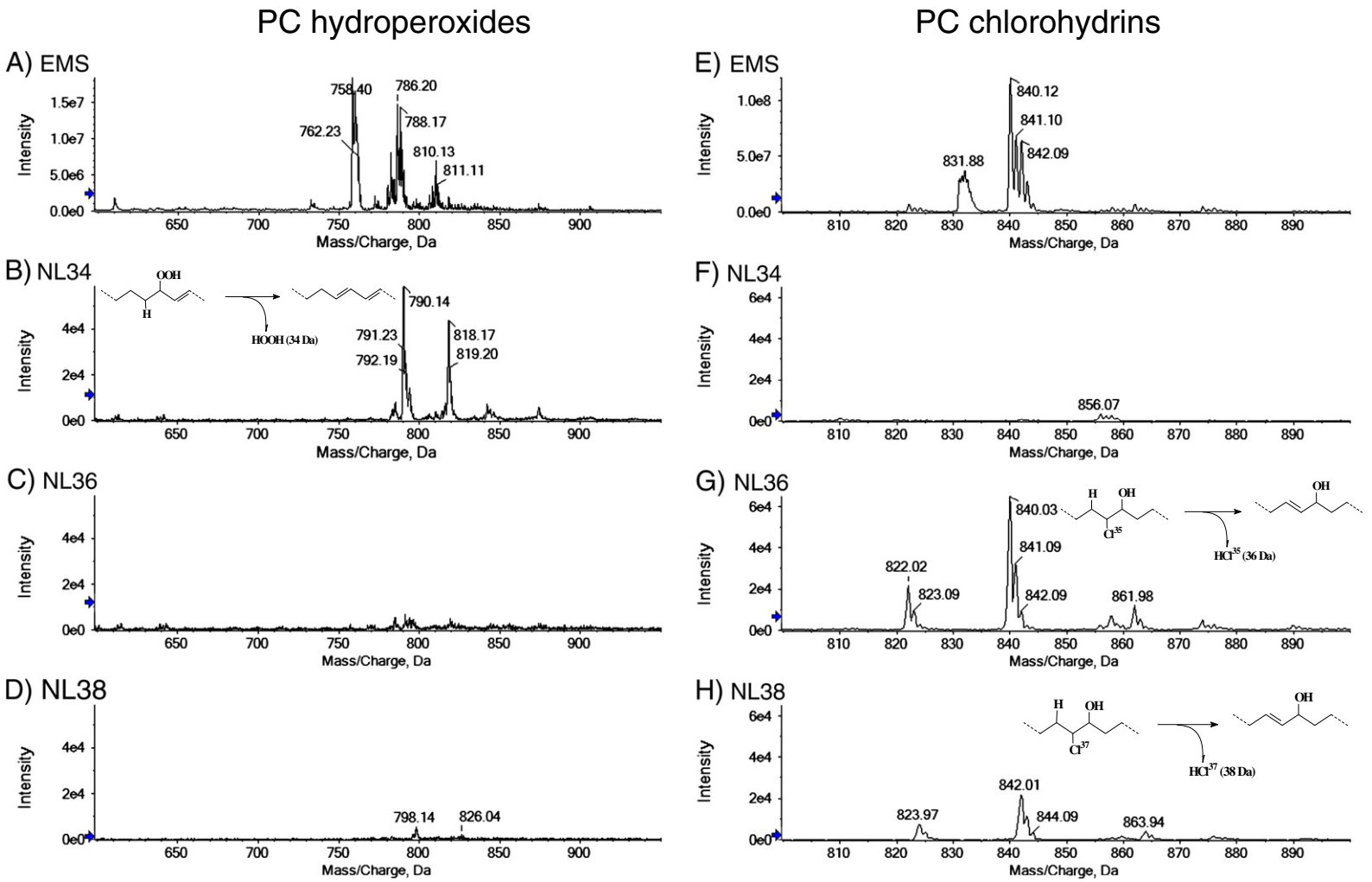

Fig. 5. Detection of (A–D) hydroperoxides and (E–H) chlorohydrins. A mixture of phosphatidylcholines was oxidized in vitro with HOCl or *tert*-butylhydroperoxide + Fe(II). All spectra were acquired in positive-ion mode at a flow rate of 3 μl/min. (A and E) EMS spectra. (B and F) Scanning for neutral loss of 34 Da, corresponding to loss of H_2O_2 and selective for hydroperoxides. (C and G) Scanning for neutral loss of 36 Da, corresponding to loss of $H^{35}Cl$ and targeting chlorinated species, although a small percentage of signal may occur from double loss of water. (D and H) Scanning for neutral loss of 38 Da, corresponding to loss of $H^{37}Cl$ and selecting chlorinated species, although a small percentage of signal may occur from loss of potassium adducts.

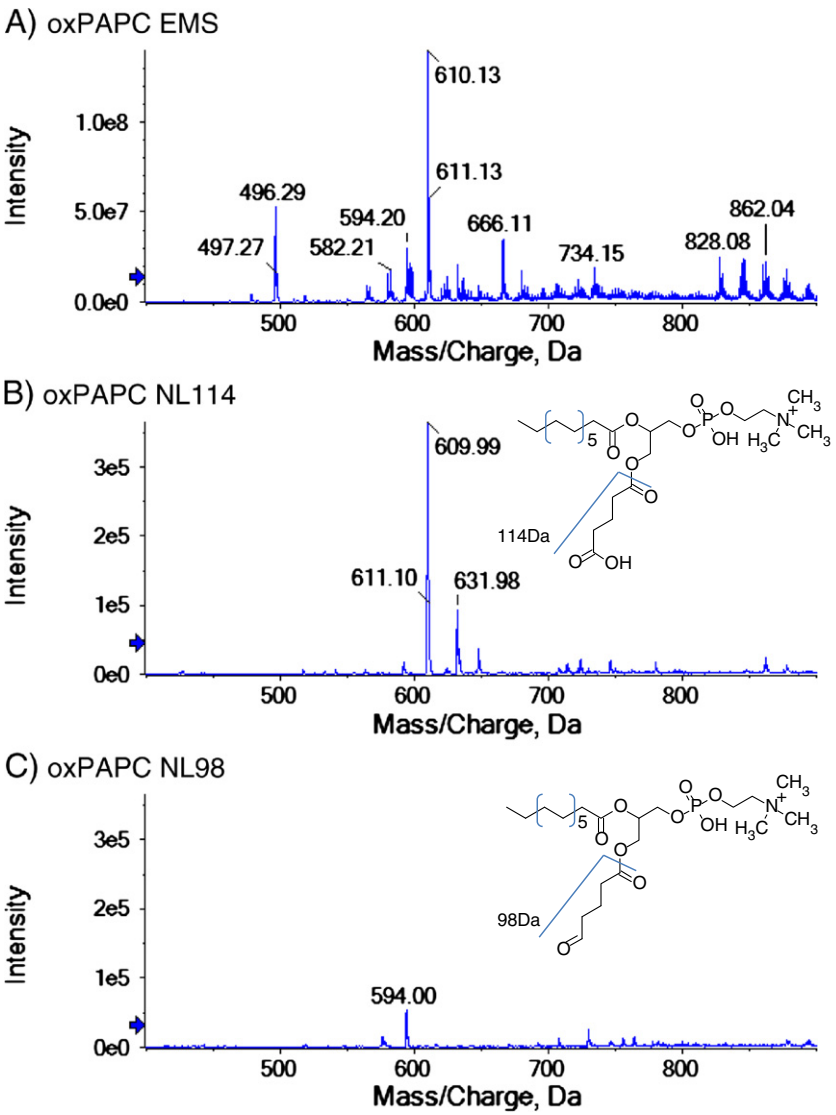

Fig. 6. Neutral loss scanning to detect individual oxidized and chain-shortened phospholipids. (A) EMS spectrum of an autoxidized PAPC sample with direct infusion. (B) Scanning for neutral loss of 114 Da, targeting molecular ions containing a glutaroyl moiety, in this case PGPC at 610 *m/z* and its sodium adduct at 632 *m/z*. (C) Scanning for neutral loss of 98 Da, targeting molecular ions containing an oxovaleroyl moiety, in this case POVPC at 594 *m/z*. The precursors appearing at higher masses are longer chain oxidation products that can also fragment to yield these moieties at high collision energy.

(790 *m/z*, eluting at 15.8 min) but SLPC hydroperoxide (818 *m/z*) and SAPC bis-hydroperoxide (874 *m/z*) were clearly observed coeluting at 17.4 min. For chlorohydrin detection, neutral loss of both 36 and 38 Da is expected, as can be seen from the similarity between traces (Figs. 8D and E). Here, the most intense signal is for the monochlorohydrin of SOPC at 840 *m/z* and the corresponding ^{37}Cl isotope peak at 842 *m/z*. The smaller signals in the chromatogram, eluting earlier, are from the complex family of chlorohydrins of PAPC (Table 3); the 938/940 *m/z* series correspond to tris-chlorohydrins, and the 920/922 *m/z* series are their dehydration products (−18 Da). The 956/958 series identified result from loss of HCl (−36 Da) from the tetra-chlorohydrin series at 990 *m/z*.

Caveats

Lipid extraction method

There are many variations on lipid extractions methods, but it is impossible to find one method that is ideal for all lipids. For extraction of phospholipids, we have used both minor modifications of the Bligh and Dyer procedure [34,40] and the Folch method [35], with good results. For extraction of phospholipids from LDL samples, we found the Folch method to give the best overall profile of lipids, compared to four other methods. However, the optimal extraction method varies dependent on the cells, tissues, or fluids used, and each laboratory will need to decide the best method for its application.

Detection and quantification by mass spectrometry

It is essential to understand that the signals observed in mass spectrometry depend on the ionizability of the analytes; compounds that do not ionize readily or with the correct polarity will give weak or no peaks in the mass spectrum. Moreover, MS instruments have a limited dynamic range, because of suppression of ionization of minor species in the source and the physical nature of the MS and detector, with standard instruments usually around 10^3–10^4 and more modern instruments, especially when using the selective ion scanning methods described here, reaching 10^5. Ion suppression is a significant complication in interpreting mass spectra when minor components of lipid mixtures are of interest; just because a phospholipid is not observed does not necessarily signify it is not present. It also means that although MS is a useful qualitative technique, great

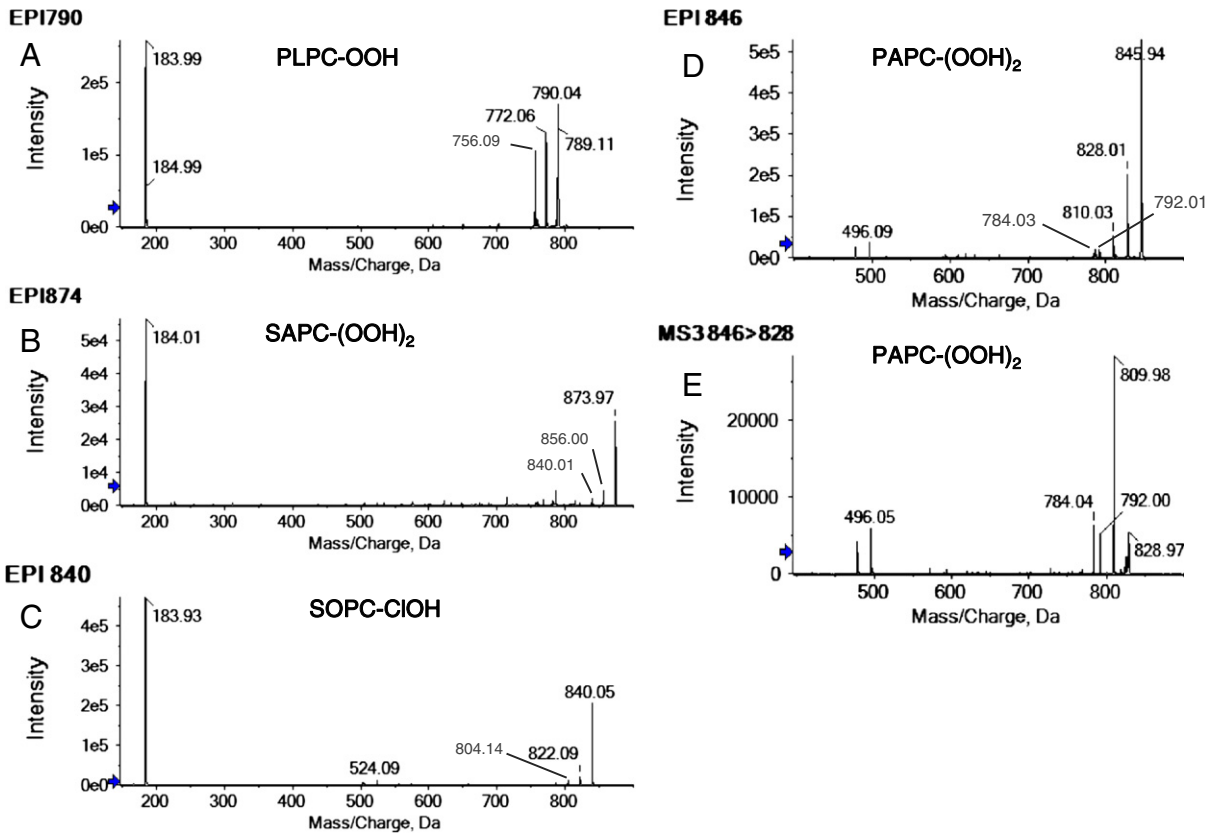

Fig. 7. Fragmentation of phospholipid hydroperoxides and chlorohydrin. Product ion scanning of (A) PLPC hydroperoxide at 790 *m/z*, (B) SAPC bis-hydroperoxide at 874 *m/z*, (C) SOPC chlorohydrin at 840 *m/z*, and (D) PAPC endoperoxide at 846 *m/z*. (E) The product ion of 846 *m/z* appearing at 828 *m/z* was fragmented further in MS^3 mode to demonstrate that the species at 810 *m/z* could occur by loss of a second water from 828 *m/z*.

care needs to be taken when generating quantitative information. Relative quantification of a particular analyte between two samples is fairly robust as long as the two samples are similar in overall composition. It is not possible to compare different metabolites by their peak intensities, as they are likely to ionize with different efficiencies, although this can be used approximately for very similar molecules. For phospholipids, even the length and degree of unsaturation of the fatty acid chains can affect the ionization efficiency, with those containing longer chains ionizing less well [25]. Absolute quantification can be achieved accurately only using internal standards. The best standards are stable isotope-labeled phospholipids, for example, a commonly used standard is phosphatidylcholines containing nine deuteriums in the choline headgroup (*m/z* 9 Da higher than the unlabeled lipids), which do not usually interfere with other signals in biological samples, although they are not currently commercially available for oxidized species. Alternatively, unlabeled phospholipids that do not occur naturally in the tissue/cell/fluid in question can be used; examples are dimyristoyl (C14:0/C14:0) or diheptadecanoyl (C17:0/C17:0) species in mammalian tissues, although even the former can be observed in some tissues. Consequently a preliminary analysis to determine the phospholipid composition of the sample and identify an appropriate standard is recommended before preparing a calibration curve for each species of interest [9].

The detection of oxidized phospholipids in tissue or cell extracts is challenging even with modern methodology, owing to the complexity of lipid profiles in cells and tissues. The levels of oxidized species are usually very low compared to native phospholipids (<1%) and ion suppression is a problem unless good separation techniques are used. The situation is further complicated by the fact that oxidized phospholipids may be metabolized or detoxified in vivo.

Structural information

Simple mass scanning to give the *m/z* ratio of the molecular ion cannot provide information about the composition of each fatty acyl chain; it can only indicate the total number of carbon atoms and double bonds in the chains. For example, a species of *m/z* 758 is most likely PLPC (16:0/18:2) but could alternatively be the isobaric species palmitoleoyl-oleoyl PC (16:1/18:1). The occurrence of isobaric structures is common when analyzing more complex mixtures of PL, especially when native and modified phospholipids are present in the mixture. These can be discriminated based on their HPLC elution profiles, as modification induces changes in the physical properties of phospholipids, facilitating their separation from the native phospholipids or from the different shortened phospholipids, such as reported previously [41] between chain-shortened oxidized phospholipids of PLPC and PAPC containing both aldehyde and carboxylic terminal moieties. To complement the elution profile data, fragmentation experiments may be needed to confirm the fatty acid composition by giving the *m/z* of the lysolipid forms when working in positive-ion mode or by giving the *m/z* of the carboxylate anions ($RCOO^-$) when working in negative mode. Ion mobility separation, which adds an extra dimension of separation in the mass spectrometer based on the size, shape, and charge of the ions, may offer an alternative approach to overcoming problems of isobaric species, although as yet there are few reports of its application to phospholipid analysis [42].

Limitations of mass spectrometry and the QTrap5500

Whereas mass spectrometry is a very powerful technique, it is not one that can be undertaken lightly by the nonexpert. Apart from the

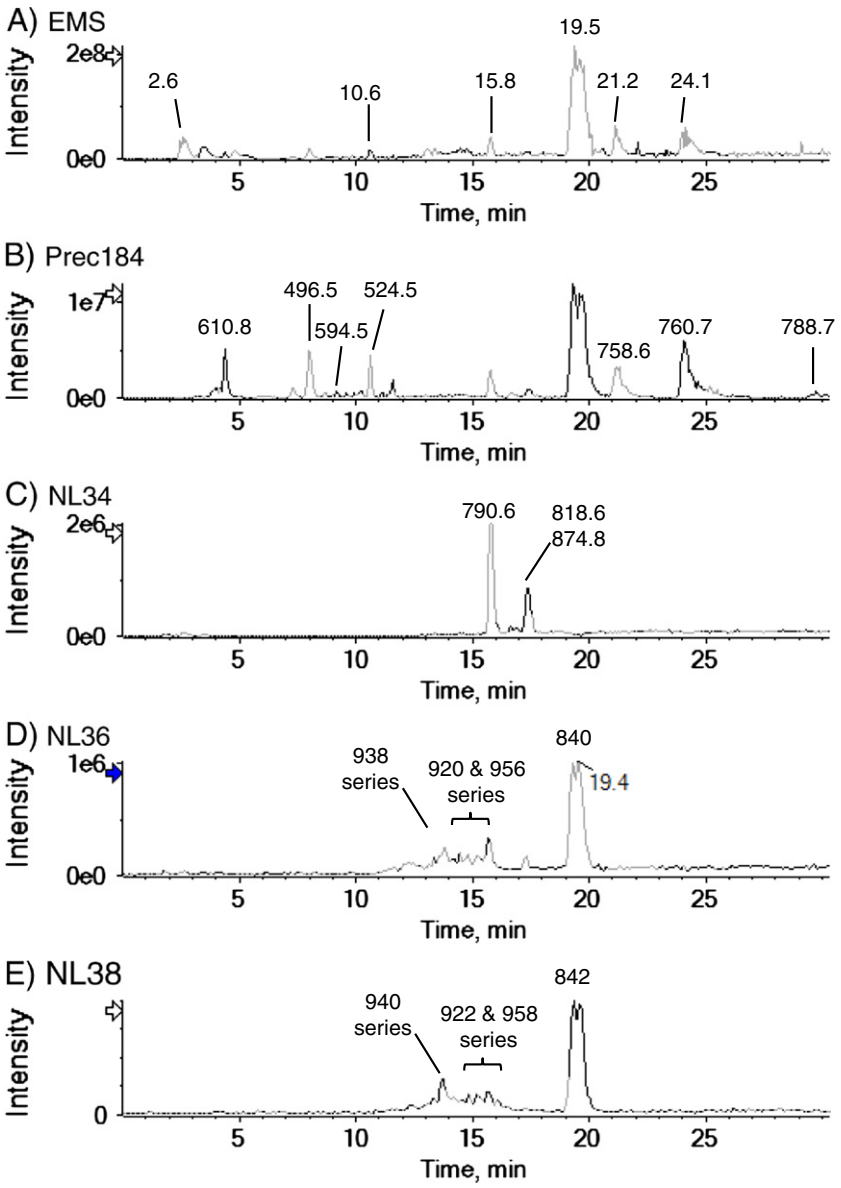

Fig. 8. Chromatographic separation and targeted detection of oxidized phospholipids with precursor ion and neutral loss scanning. The volume of sample loaded was 10 µl and the flow rate was 100 µl/min. (A) EMS chromatogram with the retention times of major peaks indicated. (B) Scanning of precursors of 184.1 *m/z* to identify the PCs. (C) Scanning for neutral loss of 34 Da to identify hydroperoxides. (D and E) Scanning for neutral loss of 36 and 38 Da, respectively; together, these identify the chlorinated species present.

fact that the instrumentation is very expensive to purchase and maintain, access to technical expertise is important owing to the complex nature of the instruments and software. Incorrect operation, including tuning and calibration, can lead to misinterpretation. As with all scientific experimentation, validation by additional methods (for example, product ion analysis or MRM protocols) is always recommended. There are a variety of geometries of mass spectrometers, which all have advantages and disadvantage as mentioned under Principles. The QTrap5500 is a very flexible and sensitive instrument, but its limitation is the mass scan range of the trap, which has an upper limit of 1000 *m/z*, and of the collision cell (1250 *m/z*). This means that some larger phospholipids, notably cardiolipin, cannot be detected in their singly charged forms. Cardiolipin can be doubly charged, as it contains two phosphate groups, but this adds a layer of complexity to the analysis. Some highly oxidized forms of phospholipids containing docosahexaenoic acid or eicosapentaenoic acid also have *m/z* ratios greater than 1000 and cannot be observed. Thus the QTrap5500 would not be the instrument of choice for these particular lipids.

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