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Computational solutions in redox lipidomics – Current strategies and future perspectives

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A B S T R A C T

The high chemical diversity of lipids allows them to perform multiple biological functions ranging from serving as structural building blocks of biological membranes to regulation of metabolism and signal transduction. In addition to the native lipidome, lipid species derived from enzymatic and non-enzymatic modifications (the *epilipidome*) make the overall picture even more complex, as their functions are still largely unknown. Oxidized lipids represent the fraction of epilipidome which has attracted high scientific attention due to their apparent involvement in the onset and development of numerous human disorders. Development of high-throughput analytical methods such as liquid chromatography coupled on-line to mass spectrometry provides the possibility to address epilipidome diversity in complex biological samples. However, the main bottleneck of redox lipidomics, the branch of lipidomics dealing with the characterization of oxidized lipids, remains the lack of optimal computational tools for robust, accurate and specific identification of already discovered and yet unknown modified lipids. Here we discuss the main principles of high-throughput identification of lipids and their modified forms and review the main software tools currently available in redox lipidomics. Different levels of confidence for software assisted identification of redox lipidome are defined and necessary steps toward optimal computational solutions are proposed.

1. Oxidized lipids and the epilipidome

Lipids are characterized by extremely high structural diversity translated to the wide range of physicochemical properties which allow them to perform different functions including organization of cellular and organelle membranes, regulation of membrane fluidity and curvature, control of cellular and organism energy metabolism, as well as being mediators in multiple signaling pathways. Such a diverse range of biological activities is attributed to the variety of lipid structures generally classified into eight categories including fatty acids,

Abbreviations: AGEs, Advances glycation end products; AIF, All ion fragmentation; CCS, Collisional cross-sections; CID, Collision-induced dissociation; CL, Cardiolipin; COX, Cyclooxygenase; cyt c, Cytochrome c; DDA, Data dependent acquisition; DESI, Desorption electrospray ionization; DGDG, Digalactosyldiacylglycerol; DIA, Data independent acquisition; EET, Epoxyeicosatrienoic acid; EpDPE, Epoxydocosapentaenoic acid; EpETE, Epoxyeicosatetraenoic acid; ESI, Electrospray ionization; FA, Fatty acid; HCD, Higher-energy C-trap dissociation; HDoHE, Hydroxydocosahexaenoic acid; HEPE, Hydroxyeicosapentaenoic acid; HETE, Hydroxyeicosatetraenoic acid; HODE, Hydroxyoctadecadienoic acid; ID, identification; IMS, Ion mobility spectrometry; LAESI, Laser ablation electrospray ionization; LC, Liquid chromatography; LESA, Liquid extraction surface analysis; LC, Liquid chromatography; LESA, Liquid extraction surface analysis; LOX, Lipoxygenase; LPPs, Lipid peroxidation products; LSI, Laser spray ionization; MALDI, Matrix assisted laser desorption ionization; MALDI-IMS, MALDI imaging mass spectrometry; MRM, Multiple reaction monitoring; MS, Mass spectrometry; NLS, Neutral loss scans; OAP, Oxygen addition product; OCP, Oxidative cleavage products; oxPAPC, Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; PAzePC, 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PGPC, 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine; PIS, Precursor ion loss scans; PLs, Phospholipids; PONPC, 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine; POVPC, 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine; PRM, Parallel reaction monitoring; PS, Phosphatidylserine; PUFAs, Polyunsaturated fatty acids; QSPR, Quantitative structure–property relationships; RPC, Reversed-phase chromatography; RT, Retention time; TAG, Triacylglycerol; TOF, Time of flight

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glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [1].

The lipidome is also subjected to different enzymatic and non-enzymatic modifications. Indeed, chemical modifications of biomolecules via the introduction of small functional groups are well known regulators of various biological functions. Epigenetic regulation of differential gene expression in various tissue and cells by DNA methylation showed its significance in the majority of biological processes and functions [2,3]. Post-translational modifications (PTMs) of proteins especially protein phosphorylation, glycosylation, and acetylation determine the variety of proteoforms (a subset of molecular species of the same protein carrying different PTMs) and are well known regulators of cell signaling in physiological and pathological conditions [4]. Recently, the concept of epimetabolites, products of enzymatic transformations of primary metabolites resulting in new functional activities, was formulated by Showalter et al. [5]. Similarly, modifications of lipids via enzymatic and non-enzymatic modifications including oxidation, nitration, sulfation and halogenation compose a new level of lipidome complexity (epilipidome) required to regulate complex biological functions.

Several excellent examples have illustrated the regulatory role of the epilipidome and lipid modifications in directing cell fate and signaling events. Enzymatically oxidized phospholipids emerge as important regulators of innate immune responses [6]. For example, lipoxygenase (LOX)-mediated oxidation in the platelet lipidome upon thrombin activation results in the formation of over 100 oxidized phosphatidylethanolamine (PE) lipids including species with pro-coagulant activities [7]. Furthermore, it has been proposed that LOX oxidation of arachidonic and adrenic fatty acyl chains in PE lipids might be associated with induction of ferroptotic cell death along with non-enzymatic lipid peroxidation [8]. Non-enzymatic or Cytochrome C mediated oxidation of cardiolipin (CL), a mitochondria specific lipid, was shown to be involved in initiation of the intrinsic apoptotic pathway [9]. Interestingly, a close cross-talk between lipid (CL) and protein (cyt c) oxidative modifications in regulation of this process was proposed recently [10]. Other well-known examples of regulatory roles of lipid oxidation include pro- and anti-inflammatory eicosanoid signaling [11] and the regulation of metabolic pathways via activation of nuclear receptors such as peroxisome proliferator-activated receptors [12]. The special role of oxidized lipids in the activation of innate immune responses via interaction with pattern recognition receptors including TLRs, CD36, RAGE, and scavenger receptors was demonstrated to contribute to pathogenesis of numerous human disorders characterized by chronic inflammation [13–15].

Despite the apparent significance in the regulation of multiple biological functions, the number of experimentally detected oxidized lipids remains relatively low. The most studied and well characterized subset of the epilipidome corresponds to oxidized free fatty acids derived from the enzymatic reactions catalyzed by LOX, cyclooxygenase (COX), and cytochrome P450 enzymes or through free radical driven oxidation of polyunsaturated omega-6 and omega-3 fatty acids [16]. However, a much smaller number of modified lipids are reported to be detected and identified *in vivo* for other lipid classes such as glycerolipids, glycerophospholipids (PLs), sphingolipids and cholesterylesters. The main challenges in addressing these subsets of the epilipidome are their low natural abundances, and the lack of knowledge about their chemical diversity in biological matrices (e.g. biofluids, tissue homogenates, cell extracts).

One can predict the complexity of the oxidized fraction of the epilipidome based on the mechanisms known for oxidation of polyunsaturated fatty acids (PUFAs; Fig. 1A). The pentadienyl moiety present in natural PUFAs is one of the main sites of lipid modification both via enzymatic and free radical reactions. Considering only four main oxidative modifications (hydroperoxy, hydroxy, keto, and epoxy groups) on a defined number of *bis*-allylic sites (Fig. 1A) in 11 PUFAs, the number of oxygen addition products (OAP) or so called long-chain oxidation products (A_n, Fig. 1B) can be estimated by enumeration. Furthermore, providing the possibility of oxidation mediated truncation via Hock cleavage and β-scission reactions initiated by the formation of lipid-bound hydroperoxide, the number of truncated or oxidative cleavage products (OCP) can be derived (C_n, Fig. 1B). Another level of complexity comes from PUFAs with ≥ 3 double bonds since such fatty acid moieties can additionally form prostane ring structures via cyclization of acyl chains. Ten types of prostanes (M_p, Fig. 1B) and at least four other combinations including IsoK and thromboxanes (M_o, Fig. 1B) are known to contribute to the diversity of this modification type (P_n, Fig. 1B). Combination of OAP, OCP and cyclization derived structures will provide the number of possible modified PUFAs (F_{ox}, Fig. 1B). One to four fatty acyl chains per lipid species ($X_{1\text{-}4}$, Fig. 1A) can be theoretically found in different classes of lipids. Thus, a very rough estimation for the number of modified lipids using 19 FA listed in Table S1 ($F = 19$, $B_0 = 6$, $F_t = 8$) without even considering the specific modification sites will provide 733 theoretically possible oxFA species (F_{ox}, Fig. 1C), which lead to 1.22×10^6 total number of oxidized lipids that contains one oxFA (T_{ox}, Fig. 1C). The total number increases further to 1.35×10^{10} if all FA residues can be oxidized (T_{ox}^{all}, Fig. 1C). Changes in FA, lipid classes, and modification types (e.g. nitration, halogenation, modifications on PL head groups, or further oxidation on prostanes) can largely influence the predicted number of possible oxidized lipids. Additionally, oxidation products from lipid classes that do not have FA residues such as oxysterols were not enumerated in these equations. Furthermore, considering modification site specific isomers the estimated number of lipid species rise to 2,241 for F_{ox}, 6.31×10^7 for T_{ox}, and 2.61×10^{13} for T_{ox}^{all}. Thus, the predicted oxidized lipidome demonstrates remarkably higher complexity than its unoxidized form (*sn* unspecific T_{unox} 1.02×10^5, *sn* specific T_{unox} 1.37×10^6). Double bond positions in FA before oxidation (e.g. omega-3, or omega-6) and possible rearrangement after oxidation including *cis/trans* form of the double bond can bring another level of complexity. Computational software to perform estimation of the oxidized fraction of epilipidome following the equations in Fig. 1B and site specific epilipidome are provided on Github (https://github.com/SysMedOs/LipidomeEstimation). The overall possible solution search space of modified lipid structures in the molecular weight range from 200 to 1500 is enormous. Thus, prior knowledge of the analytes is extremely important to generate an appropriate version of *in silico* oxidized lipidome to be used for the identification to reduce the processing time and false discovery rates.

Such structural complexity formed by addition of different functional groups will certainly determine the functional activities of oxidized lipids. Indeed, lessons learned from the structural and functional diversity of free fatty acid derived oxidation products like prostaglandins and resolvins [17], lead to the expectation of different biological activities from structurally different oxidized phospholipids (PLs). Furthermore, it is very probable that not only the structure but also the active concentration and specific tissue localization of oxPLs might determine their functional outcomes. Thus, to understand the biological significance of modified lipids on the systems biology scale, accurate and specific methods capable of high-throughput are required for “big data” acquisition. Modern mass spectrometry (MS) with its high accuracy and resolution especially when combined with other separation techniques has been shown to be the method of choice for systems-wide profiling in several omics fields including proteomics, metabolomics, and lipidomics. With some modifications, methods of structural analysis developed for metabolomics and lipidomics can be transferred to the analysis of oxidized lipids. Indeed, MS analysis allows simultaneous detection and identification of multiple molecular species present at the wide range of concentrations in complex biological matrixes. Thus, sensitivity of the current instrumentation in combination with protocols developed to ensure specificity of structural elucidations provide the opportunity to describe the diversity of oxidized lipidomes in different experimental, physiological or pathological conditions in a

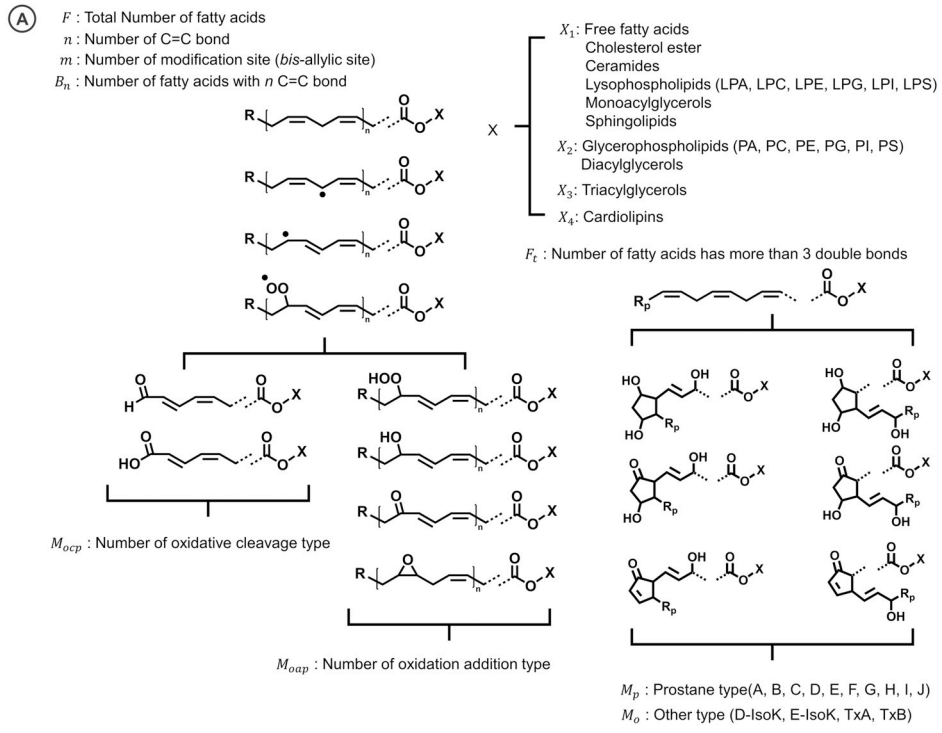

Fig. 1. Estimated number of lipid species in oxidized fraction of epilipidome for a given list of FA (F) and lipid classes (X). (A) General types of lipid modifications including oxidative cleavage products (M_{ocp}), oxygen addition products (M_{oap}), and specific modification types such as prostane like (M_p) and ring derived structures (M_o) for FA with more than 3 double bonds. (B) The equations for the prediction of oxidized lipidome from a given number of FA, lipid classes, and modification types. Reported results correspond to the unique number and combinations of modifications regardless of modification site. (C) The example of predicted oxidized lipidome using the list of 19 FAs (Table S1).

B

Number of OAP for FA with n C=C $A_n = B_n \times \left(\binom{(M_{oap}+1)+m-1}{m} - 1 \right)$

Number of OCP for FA with n C=C $C_n = B_n \times \left(M_{ocp} + \sum_{i=1}^{m-1} \left(\binom{(M_{oap}+1)+i-1}{i} \times M_{ocp} \right) \right)$

Number of other product for FA with n C=C $P_n = B_n \times (M_p + M_o) \quad n \geq 3$

Number of sum product for FA $F_{ox} = \sum_{k=1}^{n} (C_k + A_k + P_k)$

Number of sum product for Lipids With max 1 FA chain got oxidation $T_{ox} = X_1 F_{ox} + X_2 F_{ox} F + X_3 F_{ox} \binom{F+2-1}{2} + X_4 F_{ox} \binom{F+3-1}{3}$

Number of sum product for Lipids With all FA chain got oxidation $T_{ox}^{all} = X_1 F_{ox} + X_2 \times \left(\binom{F_{ox}+F+2-1}{2} - \binom{F+2-1}{2} \right) + X_3 \times \left(\binom{F_{ox}+F+3-1}{3} - \binom{F+3-1}{3} \right) + X_4 \left(\binom{F_{ox}+F+4-1}{4} - \binom{F+4-1}{4} \right)$

C

	$m_{bis-allylic}$	$m_{C=C}$	$m_{allylic}$
F_{ox}	733	1467	2844
T_{ox}	1.22E+06	2.44E+06	4.73E+06
T_{ox}^{all}	1.35E+10	2.05E+11	2.81E+12

Only *bis*-allylic sties: $m_{bis-allylic} = n - 1$
Next to each C=C bond: $m_{C=C} = n$
All allylic sties: $m_{allylic} = n + 1$

variety of biological samples and in a truly high-throughput manner. However, the bottleneck of current redox lipidomics remains the accuracy, specificity and throughput of identification strategies.

2. Redox lipidomics analytical strategies

As mentioned above, the main challenges in the analysis of oxidized lipids are their low *in vivo* abundance and diversity of physicochemical properties. To detect low quantities of modified lipids in biological matrices targeted or semi-targeted methods are often used to increase the sensitivity of the detection techniques. On the other hand, structural diversity requires the application of different analytical protocols which complicates truly Omics coverage of the epilipidome. Usually specific analytical methods addressing a subtype of oxidized lipids are developed and optimized based on the properties (concentration, polarity, ionization properties, volatility, and matrix effects) of the analyte. In terms of data acquisition strategies, lipidomics can be performed in a targeted (multiple reaction monitoring, MRM), semi-targeted (precursor ion or neutral loss scans, PIS or NLS) or untargeted (data dependent and independent acquisition, DDA and DIA) manner. Based on the aim of the study, the quantification of known lipid targets is usually achieved by targeted methods, while untargeted approaches are more favorable for lipid profiling or *de novo* identification.

Depending on the aim of the analysis, various instrument settings can be applied for both identification and quantification purposes. In general, shotgun and LC-MS-based lipidomics using electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI; including MALDI imaging) are the mainstream lipidomics strategies. In shotgun lipidomics, samples are directly introduced into an MS system without prior separation. Shotgun lipidomics provides robust and high-throughput means of lipid analysis [18]. However, identification of oxidized lipids based on the interpretation of the MS/MS spectra appears to be very challenging without prior separation due to the large number of isomeric species. Thus, shotgun methods are usually not suitable for the profiling of oxidized lipid from complex mixtures.

Targeted LC-based methods, such as MRM on triple quadrupole instruments, and more recently TOF MRM and parallel reaction monitoring (PRM), can detect selected compounds with high selectivity and sensitivity and are often used for quantification purposes. MRM-based quantification can cover a dynamic range of up to four orders of

magnitude, providing precise quantification in a broad range of biological concentrations. However, the characteristic ionization and fragmentation criteria for targeted compounds need to be specified prior to the experiment including selection of compound-specific MRM transitions (pairs of precursor and fragment ion *m/z*) and thus require prior knowledge of the analyte's structure. Additionally, despite a high degree of multiplexing, the maximum number of targeted analytes is still limited by instrument capabilities.

The development of MRM methods is generally based on well-studied fragmentation patterns and elution profiles from chemically defined authentic standards. For oxidized fatty acids, MRM transitions have already been developed and published in previous years based on over a hundred commercially available standards [19–21]. For oxysterols, which have more than 30 commercially available standards including isotope labelled internal standards, MRM transitions were also developed and reported in different use cases [22,23]. However, for lipid classes such as phospholipids, ceramides, and glycerolipids, which lack commercially available oxidized lipid standards, the applications of targeted methods are limited and less reported. To the best of our knowledge in March 2019, ten authentic standards (hydroperoxy and hydroxy derivatives of SAPC/SAPE with functional groups on carbon 15, butenoyl and azeloyl platelet-activating factor (PAF), C5 and C9 truncated PAPC with terminal aldehyde or carboxylic groups) and single isotope labelled standard (1-palmitoyl-2-glutaryl-*sn*-glycerophosphatidylcholine-d_6) were commercially available for oxidized phospholipids. To overcome these limitations, an elegant approach for the detection of oxidized PC lipids in biological samples was designed by Gruber et al. [24]. Authors used *in vitro* oxidized mixtures of PC(16:0/20:4), PC(16:0/18:2), PC(18:0/20:4), and PC(18:0/18:2) lipids to determine *m/z* values of formed OAP and OCP precursor ions which were further used to compose a list of MRM transitions using the pair of *in vitro* detected precursor *m/z* and fragment ion of PC head group at *m/z* 184 in positive ion mode. Using retention time range specific for oxidized PC (earlier elution times in comparison to unmodified lipids on reverse phase columns) as well as four commercially available oxPC standards (POVPC, PGPC, PONPC, and PAzePC), it was possible to optimize targeted methods for sensitive detection of oxidized PC lipids in a variety of biological samples [25–27].

Lack of commercial standards limits the application of MRM methods for profiling previously unknown oxidized lipids. Application of a semi-targeted method such as precursor ion scan on triple quadrupole or QTrap instruments provides a compromise solution to minimize the trade-offs between sensitivity and coverage of oxidized lipids. For instance, using precursor ion scan for the fragment ions at *m/z* 115, known to be diagnostic for the presence of hydroxy or epoxy functional groups at C5 atom in PUFA, identification of several mono- and polyoxygenated PUFAs was achieved [28].

However, the true systems-wide profiling of oxidized lipids can only be performed using untargeted redox lipidomics. The most common untargeted approaches can be divided into data dependent (DDA) and data independent acquisition (DIA) methods. A typical DDA method utilizes an MS survey scan followed by several (the number is dependent on the speed of used mass analyzer) MS/MS scans, thus resulting in a duty cycle of 1–2 s and allowing for the acquisition of several thousands of tandem mass spectra over time of chromatographic separation. Despite the large number of MS/MS data obtained in DDA experiments, the main disadvantage of the method is so called "undersampling" – selection of the signals for the fragmentation is based on their intensities (*n* signals with the highest intensity in each MS survey scan) and thus low abundant ions are not selected for MS/MS or selected only occasionally thus reducing the reproducibility of the fragmentation events and identification rates between technical or biological replicates [29–31]. The development of modern mass analyzers capable of combining high mass accuracy and resolution with high scanning rates has enabled a reduction of the effect of "undersampling". Nevertheless, DDA experiments aiming to identify oxidized lipids in complex biological samples often require separation of lipids by liquid chromatography (LC) coupled on-line to MS. LC-MS coupling usually employs separation of lipid mixtures based on reverse phase (RP) or hydrophilic interactions liquid chromatography (HILIC) techniques [32] and allows to reduce sample complexity at the moment of ionization (reducing ion suppression) as well as the number of ions competing to be selected for the fragmentation events. For instance, combination of two orthogonal chromatographic techniques (normal phase and reverse phase) in combination with DDA was used for the analysis of oxidized phospholipids (CL, PE, PC, and PS) in four different cell and tissue models of oxidative stress [33]. Analysis of the lipidomes of rat cardiac cells treated with peroxinitrite donor by RPLC-DDA MS allowed identification of 67 oxidized PLs including PC, PE, PG, PS, and PA lipids [34].

Selection of appropriate stationary and mobile phases for LC separation is crucial to ensure coverage of the lipidome [35] and epilipidome [7]. Thus, using a combination of two different RPC methods optimized for non-polar and polar analytes combined with untargeted MS analysis in positive and negative ion modes as well as two different *m/z* ranges, Slatter et al. performed an in-depth analysis of the platelet lipidome upon thrombin activation [7]. This analytical platform allowed identification of over 100 putative oxidized PE lipids, which were further verified in DDA experiments and relatively quantified using MRM assay. Importantly, large amounts of data obtained in this study and thus identification of potential oxPLs signals was performed using an in-house designed Excel tool for peak peaking, deconvolution, retention time correction and noise reduction which was further translated into the open-source software LipidFinder [36].

An interesting approach for oxPL identification and characterization of their fragmentation patterns was performed by Aoyagi et al. [37]. Authors used biogenic conversion of commercially available oxidized fatty acids (HETE, HEPE, HDoHE, HODE, EET, EpETE, and EpDPE) by HEK293 cells into their corresponding oxidized phospholipids. Extracted lipids were analyzed by DDA LC-MS/MS which allowed identification of 386 oxPL species that were further used to build oxPLs spectra libraries as well as to optimize MRM transitions for the targeted analysis. Furthermore, application of defined positional isomers of oxidized PUFAs (e.g. 12-HETE) enabled the definition of position specific fragment ions, significantly improving the specificity of the identifications.

DIA acquisition overcomes the intrinsic limitations of the duty cycle length vs. number of MS/MS scans characteristic for DDA methods. For instance, the duty cycle in the All Ion Fragmentation (AIF) and MS^E method includes just two scans – one MS and one MS/MS – which allows for the collection of a large number of data points for MS-based quantification. Furthermore, DIA avoids an intensity-based selection of precursors, and all ionizable analytes eluting from the column are subjected to the CID during MS/MS scans. However, in comparison to DDA data structure, where a direct and clear connection between precursor and corresponding fragment ions already exists at the level of data acquisition, DIA data requires application of post-processing algorithms aiming to assign precursors to the corresponding fragment ions. Unfortunately, such an assignment does not currently provide a clear precursor-fragment ion association in lipidomics datasets. The presence of multiple signals corresponding to the fragment ions of other precursors interfere spectra annotation and lipid identification in manual and especially the automated software interpretations. Furthermore, assignment of the fragment ions to the corresponding precursors for structurally similar analytes can be ambiguous due to the presence of the same fragment ions (e.g., signal at *m/z* 184 will be present in all tandem mass spectra of PC lipids acquired in positive ion mode, or signal at *m/z* 303 will be characteristic for MS/MS of lipids containing arachidonic acid residue when analyzed in negative ion mode). Thus, All Ion Fragmentation (AIF) and MS^E methods are not currently used for the identification of oxidized lipids, to the best of our knowledge.

Another DIA approach known as sequential window acquisition of all theoretical fragment ion spectra (SWATH), relies on the combination of an MS survey scan followed by the sequence of consecutive MS/MS events with relatively wide isolation windows (usually 25 Da) within one instrument duty cycle. Thus MS/MS information for all precursor ions present at MS level is acquired and can be matched to the analytes using preexisting fragmentation libraries. This approach was recently applied for detection and relative quantification of four oxidized lipids (POVPC, PGPC, PAzePC, PONPC) in the platelet lipidome of patients with coronary artery disease [38]. However, detection of oxPCs required previous knowledge of their fragmentation patterns used for the spectra matching as well as optimization of SWATH method by narrowing down Q1 isolation window to 5 Da and enhancement for fragment ion at *m/z* 184 to ensure the identification accuracy. Thus, authors referred to this as method as MRM-like SWATH rather than as a truly untargeted DIA method [38].

In addition to LC-MS and shotgun MS approaches, several matrices for lipid analysis on MALDI instruments have been reported, allowing for the fast identification of lipids in both a positive and negative ionization mode. Generally, due to the complexity of the (oxidized) lipidome in biological samples, MALDI is less used for lipid profiling. However, the MALDI imaging (MALDI-IMS) technique has its unique feature to provide visual information of lipid spatial distribution in a tissue sample. Whereas MALDI-IMS of oxidized lipids represents a promising emerging direction towards the understanding of physiology and disease, as it allows monitoring of the distribution of lipid peroxidation products (LPPs) in tissues [39], oxPL imaging appears to be challenging due to the low intensities of modified lipid signals already at MS level (low natural abundance of oxPLs plus limited sample availability) limiting their confirmation by tandem MS. Although a number of methods for mass spectrometry imaging are available (based on MALDI, DESI, LAESI, LSI, LESA) [40–44], only a few studies describing the spatial distribution of oxidized lipids have been reported so far. For example, Sparvero et al. [45] reported the use of imaging mass spectrometry for lipidomics studies on traumatic brain injury, highlighting the intriguing possibility of monitoring oxidized species. Later, Stutt et al. [46] developed a MALDI MS/MS method that enabled imaging of truncated carboxylate-containing PC in rat spinal cord, proving that the ions related to these species appear mostly in the gray matter. In recent years, developments in MALDI-IMS instruments have introduced the capability of sub-micrometer sphere resolutions, enabling further applications on the cellular level [47]. However, the low abundance of LPPs still prevents wide application of imaging mass spectrometry in this field.

In summary, over the past decades the development of new LC and MS instruments has brought significant enhancement for the separation and detection of lipids. Based on new stationary phases for LC, high resolution, accuracy, speed, and sensitivity of MS instruments, various methods optimized for different lipid classes have been published [48–54]. Some methods specifically tuned for oxidized lipids have also been developed [48,54]. Still, the unknown diversity of oxidized lipids adds additional challenges not only to their analysis but to high-throughput identification strategies as well. As a result, manual interpretation of tandem spectra is usually required for the confident identification of oxidized lipid species.

3. Bioinformatics strategies for identification of oxidized lipids

3.1. Software solutions for identification of unmodified lipids

Despite a significant development in experimental designs and data acquisition strategies for MS-based lipidomics, overall data analysis is one of the most important parts, as it largely influences the outcome of the entire experiment. Additionally, the application of a new generation of high-resolution instruments produces more complex data, which often requires significant effort in data processing using advanced software tools. However, in contrast to other “omics” studies, such as genomics and proteomics, software tools in lipidomics are less developed to give a universal all-in-one solution. Untargeted (redox)lipidomics approaches are usually based on DDA datasets and aim first of all to identify as many lipid species as possible to reveal the complexity of different lipidomes. In the field of oxidized lipids, the exact structural assignment is important for further analysis of biological functions. Thus, identification methods based only on the exact mass of the analyte are not sufficient to report the presence of oxidized lipids. The automatic identification of oxidized lipids based on the acquired tandem mass spectra is the fundamental procedure of high-throughput computational workflows. Additionally, MS survey scan information about precursor mass, isotope pattern, elemental composition and retention time are used to improve the assignment depending on the software algorithms. As in many other fields, redox lipidomics tries to adapt currently available bioinformatics solutions to lipidomics for the high-throughput identification of oxidized lipids.

Current bioinformatics solutions in lipidomics are represented by three main approaches – spectra matching, top-down and bottom-up based lipid identification (Fig. 2). Lipid identification based on the scored match between experimental data and reference spectra libraries is probably the most popular approach in MS-based lipidomics (Fig. 2A). Several types of libraries are available, including MS/MS spectra (e.g., METLIN) and lipid structures (e.g., LIPID MAPS, HMDB) [55–57] based. Both types can rely on the experimental data, or on a combination of experimental and computationally-predicted entries. For instance, the LipidBlast software reports 120,000 distinct compounds (including over 33,000 of PLs, usually 5,476 species per class derived by permutation of 74 discrete FA residues) associated with more than 200,000 *in silico*-generated tandem mass spectra [58]. Such a high number of computationally predicted structures allows for the identification of previously undetected species but requires strict control of identification results. The accuracy and coverage of library matching strategies rely on the number of spectra collected in the library as well as the MS/MS quality. Furthermore, spectra matching tools are inherently instrument and acquisition type dependent [58,59]. The total number of identifications is restricted by the number of spectra included in the library, however the creation of a given spectra library is often a time-consuming task, and greater efforts are required for the maintenance and subsequent expansions to new compounds and/or other instruments.

Among the most used software tools based on a similarity matching are LipidBlast and MSDIAL tools [58,59]. Furthermore, Lipostar, a software based on a cheminformatic approach, was recently developed [60]. In Lipostar, lipid fragmentation is based on a collection of experimental fragmentation rules from literature and in-house data. The library of fragmentation rules can be used to generate *in silico* fragment databases of lipid structures (provided by the user) and subsequently to apply the spectral match strategy. Although Lipostar does not directly estimate the intensity of theoretical MS/MS fragments, several weights and labels (e.g. “mandatory” or “recommended” fragments) can be adjusted by the user to suit the software to the used experimental conditions, and fragmentation rules established by the user can be also used for *in silico* fragmentation.

The existing *in silico* top-down fragmentation algorithms for small molecules, such as MetFrag and *in silico* identification software (ISIS), can be used for certain lipid classes [61,62] (Fig. 2B). These types of algorithms require an exact structure of the compound to estimate chemical bond energies and other properties, which are further used for the prediction of cleavage sites under fragmentation conditions. Due to the high structural diversity among lipid classes, algorithms optimized for certain lipid classes might not be suitable for other lipid classes. Additionally, fragmentation algorithms are often unable to provide the relative intensities of the predicted fragments. The relative intensities of the main fragments still need to be determined using external sources, which mainly require previous knowledge of the main fragmentation

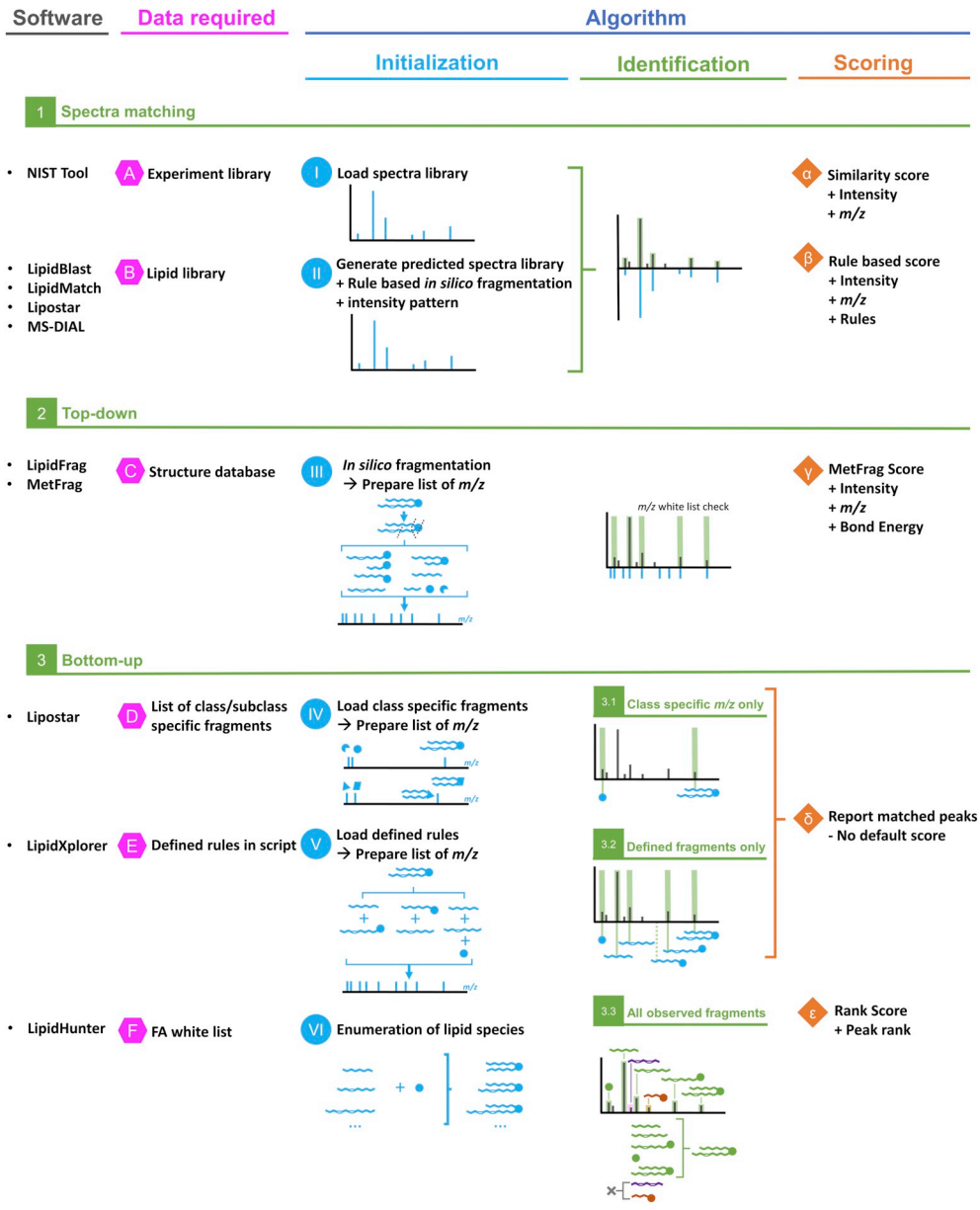

Fig. 2. Summary of identification approaches used in high-throughput MS based lipidomics including spectra matching, top-down, and bottom-up strategies. Software tools based on the spectra matching approach require an MS/MS spectra library (A, I) or lipid library (B) as input to generate MS/MS spectra using software embedded *in silico* fragmentation algorithms (II). The acquired spectra are then matched to the spectra libraries and identifications are scored based on the similarity using classical (α) or modified similarity scores (β) algorithms. Software tools which rely on the top-down identification approach require the input of lipid structure database (C) to perform *in silico* fragmentation based on chemical properties of the input structures (III) following by scoring based on *m/z*, intensity, and bond energy (γ). For bottom-up strategy, a high throughput assignment on the lipid class/subclass level can be obtained by using list of class/subclass specific fragments (D). While on the lipid species level, a highly customized workflow (E) based on user defined matching patterns (V) is used. Despite the algorithms that report the matched peaks without default score system (δ), a bottom-up identification based on a list of defined FA (F) to enumerate all possible lipid species (VI) can be used and supported by the ranking score of matched fragment ions from all identified signals (ε).

pattern of target lipid classes given the chosen instrument setup.

The bottom-up identification methods suggest most probable structures by assembling identified fragments matching precursor elemental composition (Fig. 2D). Thus, LipidXplorer [56], can utilize highly customized scripts to define fragment ions from a limited range of precursor elemental compositions. This strategy can also be adapted to define the fragment ions for a broad range of lipid classes. However, the current version of LipidXplorer was designed for shotgun lipidomics, and needs additional tuning of the configurations and workflows to be fully functional for LC-MS datasets [63,64]. Lipostar provides a bottom-up high-throughput strategy for lipid identification as well, which is applied only to those features that remained unknown after the above-mentioned spectra matching approach. Indeed, for unknown compounds, the experimental MS/MS spectrum is searched for a list of lipid class/subclass specific fragments collected in the library of fragmentation rules. If matches with specific fragments of a given lipid class are found and retention time is compatible, the unknown compound is identified as a member of that lipid class, but the elemental composition of the precursor nor scoring functions are available in the present version of the software.

The algorithm of LipidHunter, a recently developed tool for bottom-up identification of phosphor- and glycerolipids from DDA LC-MS/MS datasets, relies on three main steps [65]. First, the observed MS/MS spectrum is searched against all possible *m/z* values for lipid class and fatty acyl chain specific fragments. Second, all identified fragments will be ranked and scored. Finally, the identified fragments are summarized to suggest the best matches that fit to the elemental composition of the precursor. For bottom-up methods, the correct identification of the elemental composition is very important. Thus, the high resolution of MS spectra and high-quality isotope patterns are essential. Although no spectra library is needed in bottom-up strategies, prior knowledge of the lipid class and acyl chain specific fragmentation patterns is required. The quality of the bottom-up identification largely relies on the fragment ion signals observed in MS/MS spectra and the corresponding score system. Considering the vast possibilities of fragments from oxidized lipids and potential isomeric species, the generation of fragments list is critical for the identification accuracy.

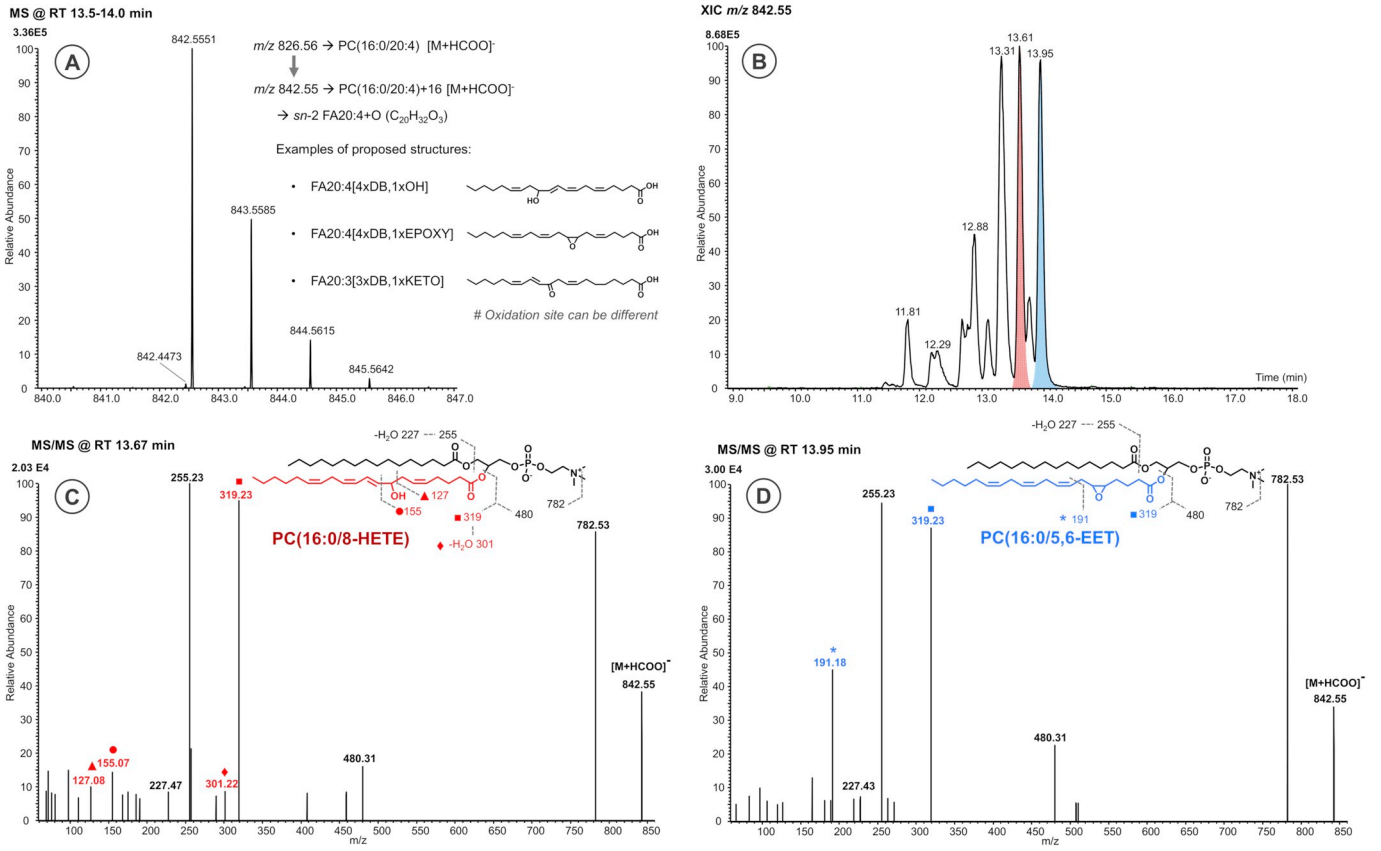

Fig. 3. LC-MS/MS derived identification of two isomeric oxidized PC lipids (PC(16:0/8-HETE) and PC(16:0/5,6-EET)) detected as $[M+HCOO]^-$ ions at *m/z* 842.55 supported by three levels of confidence including high-resolution MS survey scan to deduce lipid elemental composition (A), reverse phase separation of isomeric species (B), and modification type- and site-specific identification based on MS/MS data (C and D).

3.2. Specific requirements for identification of oxidized lipids

Identification of oxidized lipids from high-throughput DDA experiments to a certain extent follows the general logic of unmodified lipid identification but also requires several specific steps (Fig. 3). Thus, to confirm identity of oxPL, the precursor ion elemental composition needs to deduce from the *m/z* of the corresponding MS survey scan (Fig. 3A). Analysis of oxidized lipids usually requires high mass accuracy and resolution in order to distinguish oxPL species from the signals of closely isobaric unmodified lipids. For example, deprotonated ions of unmodified PS(18:0/20:4) and oxidized PE(18:0/22:4) with single hydroxy group are detected at *m/z* 810.5285 and 810.5649 (Δ m 0.0364), respectively. To distinguish these two lipids, an instrument with a mass analyzer capable of resolution (defined here as m/Δm) more than 20,000 at *m/z* 810 is required (810/0.0364 = 22,253).

Combination of MS analysis with reverse phase chromatography (RPC) separation provides another level of confirmation in detection of oxidized lipids. Due to the presence of oxygen-containing functional groups (e.g. hydroperoxy, hydroxy, epoxy, and keto moieties) in acyl chains, oxidized lipids become more polar in comparison to their unmodified counterparts, and thus elute earlier in RPC applications. Moreover, optimized chromatographic separation allows not only to distinguish oxidized from unmodified lipids by their retention times but also to separate numerous isomers of oxidized lipids (Fig. 3B). Indeed, lipid with a single elemental composition, e.g. $C_{44}H_{80}NO_9P$ detected in negative ion mode as a formate adduct at *m/z* 842.5547$^-$, can correspond either to PC lipid with palmitic acid and arachidonic acid modified to hydroxy or epoxy derivatives as well as keto derivative with the loss of one double bond. Furthermore, considering that singly oxygenated arachidonic acid can be represented by at least six hydroxylated positional isomers (e.g. 5-, 8-, 9-, 11-, 12-, and 15-HETE) and four isomers of epoxy derivatives (5,6-, 8,9-, 11,12-, and 14,15-EET), overall ten isomeric species can be expected. Based on the previous experiments on free eicosanoid standards, elution orders for functional (e.g. OH before epoxy) and positional (e.g. isomers with functional groups closer to ω-end of acyl chain elute earlier) isomers was established and showed to hold for PL-esterified oxidized fatty acids as well. Thus, availability of high-resolution and mass accuracy MS data when combined with LC separation allows putative identification of oxPLs. However, only confirmation at MS/MS level based on the presence of specific fragment ions can provide structural confirmation of LC separated isomeric oxPLs.

oxPL MS/MS spectra provide information both on the lipid class and fatty acyl chains as well as on the type and sometimes the position of modification (Fig. 3C and D). Similar to the identification of unmodified lipids, specific neutral losses and fragment ions are used to define lipid class both in positive and negative ion modes. However, identification of modification type and especially position of the functional group usually requires tandem MS experiments performed in the negative ion mode. Collision-induced dissociation (CID) and higher-energy C-trap dissociation (HCD) fragmentation of oxPL results in the formation of intense fragment anions corresponding to the fatty acyl chains esterified on the PL backbone. However, the presence of the oxidized fatty acyl chain anion is not sufficient to determine the type and position of the modified groups. Fortunately, specific neutral loss and fragment ions are usually detected to assist more detailed identification. For the example provided in Fig. 3C and D, the type of the modification (OH vs epoxy) can be distinguished by the presence (Fig. 3C) or absence (Fig. 3D) of the fragment ion formed via water loss from hydroxy derivative of arachidonic acid (*m/z* 301.3), whereas the epoxy derivative although characterized by the same fatty acyl anion (*m/z* 319.2) does not provide this water loss. Furthermore, the specific position of the

Table 1
Summary of currently available solutions for identification of oxidized lipids.

Evidence Level	Approach	Available solutions	References
MS1: matched m/z values, exact mass and/or elemental composition	Publicly available databases of lipid structures	Lipid Maps (246 oxPLx)	[49]
	Matching to in-house generated or published libraries	Previously published *m/z* values and elemental compositions of *in vitro* oxidized lipids	[45–47]
	In silico generated libraries	LOBSTAHS	[60]
	Matching with *m/z* calculated on the fly for potentially oxidized lipids from libraries of non-oxidized species	Lipostar (high-throughput)	[53]
RT: matched retention times or retention time range	Knowledge based, experimental observation (e.g. higher polarity of oxPLs relative to unmodified lipids)	Lipostar (rule-based filter – oxPL RT < PL RT)	[53]
MS2: assigned fragment ions and neutral losses specific for: • lipid class and at least one of the fatty acyl chains • modification type • modification position	Experimental MS/MS spectra libraries	MS-DIAL using MS/MS spectra libraries from the lipidome of cells treated with fatty acid precursors of oxPLs	[30]
	In silico generated MS/MS spectra libraries	Lipostar CEU mass mediator LipidMatch	[53,59,61]
	Top-down or/and bottom-up ID using integrated rules (with optimized *in silico* generated MS/MS spectra libraries)	LPPtiger	[29]

modification group can be determined by low molecular weight fragments formed via gas phase fragmentation-induced cleavage of the oxidized fatty acyl chain usually next to the position of the functional group (e.g. *m/z* 155 for hydroxy group at C8 position). Overall, such kinds of observations derived from experiments performed on free eicosanoids standards as well as on *in vitro* oxidized PLs, allowed the accumulation of a set of rules summarizing specific neutral losses and fragment ions for different types of oxidized PLs [66].

In summary, highly specific identification of oxidized lipids requires confirmation on at least three different levels – MS1, retention time, and MS/MS data including lipid class, fatty acyl, modification type and position specific fragments (Table 1). Unfortunately, although often applied to the manual analysis, this strategy has not been fully automated so far to provide truly high-throughput specific identification. Several partial solutions for automated and semi-automated identification of oxidized lipids are currently available and will be discussed in the following section.

3.3. Software tools available for identification of oxidized lipids

Several available solutions provide identification of oxPLs at the three evidence levels mentioned above (MS, RT, and MS/MS). Publicly available lipid databases contain a number of oxidized lipids with corresponding structures, elemental compositions and exact masses which can be used for the identification of oxPLs based on the mass of detected species (MS level). LIPID MAPS currently contains 246 entries for oxidized glycerophospholipids including 48 oxPC, 51 oxPE, 36 oxPS, 36 oxPI, 36 oxPA, 36 oxPG, and 3 oxCL, most of which are calculated by enumeration of C4, C5, C7, C8, C9, C12 *sn*-2 truncated chains with terminal aldehyde or carboxylic functions to PLs with different head groups and palmitic or oleic acyl chains in *sn*-1 position. Exact matching of measured *m/z* values derived from high-resolution MS experiments with database entries, can be used for putative oxPLs identifications especially when supported by RT data.

The other approach to match observed *m/z* values to potential oxPL structures at the MS1 level was used by Chen et al. [53]. Authors used previously published data on *m/z* values and elemental compositions for oxPLs obtained by *in vitro* oxidation of lipid standards [52,54] to compare with their experimental data on LC-MS analysis of zebrafish plasma in type 2 diabetes mellitus model, which allowed identification of PC-bound aldehydes (truncated lipids) as well as PC and PI peroxides (long chain oxidation products). However, the exact identification of oxidized species at the MS/MS level was not provided, thus limiting the specificity of the identification of modification types and positions of functional groups.

Recently, the software tool **LOBSTAHS** (lipid and oxylipin biomarker screening through adduct hierarchy sequences) was developed to match lipids and their oxidized forms based on the empirically defined hierarchy of ion adducts intensities [67]. The MS1 values library included 335 fatty acids and correspondingly enumerated lipids from other lipid classes considering the presence of up to four oxygens in one fatty acyl chain. Authors identified multiple oxidized lipids in control and H_2O_2-treated marine diatom algae lipidome including oxPLs (PC, PE, PG), oxTAGs, and algae specific lipids like DGDG. Unfortunately, most of the parameters for oxidized lipids were directly inherited from their unmodified counterparts including retention time and adduct formation. Thus, the specificity of adduct formation for short chain carboxylated oxidized acyl chains for PC lipids was not considered. Due to the presence of terminal carboxyl functionality, such kind of oxPCs are represented only by deprotonated anions and not as formate or acetate adducts typical of other PC lipids in negative ion mode. Furthermore, oxidized lipids due to increased polarity of the acyl chains should elute earlier from a reverse phase column and thus the retention time window for their identification should be shifted in comparison to unmodified species. Furthermore, since the identification was done at MS1 level, no details on isomer specificity were provided.

Understanding the importance of MS/MS-based structural characterization of oxidized lipids, several software tools were developed or optimized for oxPL identification. Among the software tools for high-throughput untargeted lipidomics, **Lipostar** was a pioneer in realizing that the search for oxidized species should be included in its workflow, due to the increasing importance of redox lipidomics [60]. However, the inclusion of a module to search for oxidized lipids in Lipostar was far from trivial. In a first attempt, the authors wanted to make use of the Lipid Builder module in Lipostar (a tool for automatic generation of lipid structures to be fragmented for customized generation of theoretical fragment libraries) to build databases of oxidized species. However, this approach appeared immediately too slow to fit the general use of the software in high-throughput approaches. Therefore, there was the clear need to reduce complexity and in the present version of Lipostar a very simple and fast approach is applied to extract potential oxidized species from complex lipid mixtures. Briefly, in order to reduce the computational time, the search for oxidized species occurs in a second step of identification. In a first step, features are identified taking into account non-oxidized species only, looking for matches between experimental and predicted fragmentation (with or without the use of a database of fragmented lipids). After the first identification run, the search for potential oxidized species is applied only to the remained non-identified features. To avoid the generation of libraries of fragmented oxidized species and to be able of provide results in a short

time, a simple rule-based approach is used in Lipostar:

1) When a database of fragmented lipids is used for identification, unidentified *m/z* entries are further tested as oxidized forms (M + O, M + 2O, M + O-2H etc.) of lipids in the database, based on the exact masses comparison.
2) When potential oxidized species are identified based on mass matching, the MS/MS spectra, when available, are automatically inspected for compatible increments of fragment ions *m/z*.
3) Optionally, the user can reduce the FDR by applying filters, for example requiring that for each potential oxidized lipid identified the sample must contain the corresponding non-oxidized form. This constraint can be reinforced requiring that the retention time for oxidized species should be lower than that of the non-oxidized form considering samples were separated by RPC.

Of course, this simple method is far for being exhaustive, and provides a very preliminary inspection of MS/MS data. The major drawback of this approach is that discrimination among isobaric oxidized species originated from the same lipid is not possible. On the other hand, this approach allows to pick potential oxidized species in the minutes scale.

CEU mass mediator, a web base tool for metabolites annotation by quiring different databases, recently provided a semi-automated solution for identification of oxPLs [66]. Based on the LC-MS/MS experiments using oxPAPC standards, the authors defined and validated the fragmentation rules of oxidized PLs including modification type specific neutral losses and fragment ions, some of which were implemented in CEU mass mediator module for oxidized lipids. The input data should include *m/z* values of a precursor ion (MS1), and two fatty acyl chain anions (MS2) as well as corresponding mass tolerance. For the identification of long chain oxPL species, the software substitutes the mass increments of possible oxidative modifications (e.g. OH, keto, OOH groups) from *m/z* values of acyl chain anions and tries to fit the resulted value to the list of known unmodified fatty acyl chains. If the match is found, the tool reports the identified oxPL with corresponding elemental composition, parent (unmodified) lipid as well as potential neutral losses specific to the modification type which can be manually verified by the user from raw data.

Another example of software dealing with oxidized species is represented by **LipidMatch** [68], an open source R-based tool for lipid identification from LC-MS data. The identification process is based on the use of *in silico* generated fragmentation libraries for over 250,000 lipids including, among the others, oxidized species. To generate oxPL libraries the authors predicted 214 potential oxidized fatty acids including 126 long chain (by enumeration of up to four oxygens to 39 unmodified fatty acids) and 88 short chain oxidation products (introducing the cleavage sites on unsaturated fatty acids and adding carbonyl or carboxyl terminal groups). By enumeration of all possible combinations the final list of oxPLs consisted of 214 species for LPC and LPE, 31,112 species for PC and PE, and 156,102 oxCLs, compromising over 87% (!) of lipids from a total 250,000 species library. For each oxPL *m/z* values for fragment ions specific to lipid class and acyl chains are provided. Unfortunately, specificity of the ionization for different short chain PC lipids was not considered and format adducts in the negative ion mode data were calculated for both carbonyl and carboxyl terminal oxPCs.

Simple computational enumeration methods, although providing a large library of potential species, do not consider a probability of biological existence of predicted oxPLs. To overcome this limitations, evidence-based approach for prediction of oxidized lipids was implemented in **LPPtiger** software [34]. To build the algorithm for *in silico* oxidation of PLs, the meta-study of publicly available data (over 170 publications) was performed to define rules of enzymatic and free radical driven oxidation of PUFAs. Based on this information, knowledge of the oxidation mechanisms for bis-allylic positions, rearrangement of neighbouring double bounds, cyclization to prostane ring structures, and truncation via Hock cleavage or β-scission in PUFAs were translated into an *in silico* oxidation algorithm. LPPtiger can perform *in silico* oxidation for any provided PL lipidome as long as defined acyl chains in unmodified parent PL are provided (e.g. PC(16:0/20:4)). The generated library of oxPLs is further used to perform *in silico* CID fragmentation considering oxPL specific ionization and fragmentation properties. Fragment library is used to perform similarity-based matching with experimental MS/MS spectra providing spectra similarity score based on reverse dot-product algorithm, similar to all spectra matching lipidomics software tools. However, due to the large diversity of oxPLs structures LPPtiger additionally integrated other scoring algorithms including the rank score developed in LipidHunter's bottom-up strategy [65]. Furthermore, a Fingerprint Score based on the intensity-independent matching of neutral losses characteristic to oxidized fatty acyl chain anions was introduced. Fingerprint score allows to distinguish some of potential isomers derived from different modification types – e.g. hydroxy (characterized by a water loss) vs epoxy (no water loss). Although LPPtiger provide certain level of oxPL specificity (modification type), the identification of position specific isomers (e.g. 5- vs 12-HETE-PL) is still missing.

4. Future directions to improve high-throughput identification of oxidized lipids

4.1. Improving specificity of oxPL identifications

In the previous sections the crucial role of software tools to assist high-throughput lipidomics was described, but a number of limitations to their use in the identification of oxidized lipids was also highlighted. Indeed, the correct identification of isomers are critical for further data analysis (e.g. pathway analysis) but challenging for the software identification. Due to the various possible combinations of oxidized moieties, the same elemental composition can be shared by large number of isomers. However, before asking how a software for redox lipidomics can be improved, one should ask how the experimental information on oxidized lipids can be increased, as it is evident that good software cannot be created without a strong experimental background on the topic.

Unfortunately, the information about the fragmentation of oxidized lipids is still limited. First of all, the number of oxidized lipid standards available so far is limited and available data on fragmentation patterns are usually based on in house prepared mixtures of *in vitro* oxidized lipids. Increasing the number of chemically defined pure standards for oxidized lipids will be a valuable strategy to increase a dataset necessary to build up new algorithms. Additionally, analytical (new stationary phase for separation of isomeric oxidized lipids) and instrumental (sensitivity) improvements would facilitate generation of high-quality datasets.

MS/MS spectra acquired using fixed collision energy are generally not sufficient to assign the specific modification positions and difficult to confirm the exact *sn*-1/*sn*-2 positions of oxidized FA chains. Application of stepped CID and HCD fragmentation protocols based on the combination of lower (to generate lipid class specific fragment ions) and elevated (to induce modification-driven fragmentation on oxidized fatty acyl chain anion) collision energies may generate more informative MS/MS spectra, especially in the lower *m/z* region. The MS^n techniques based on consecutive tandem mass spectrometry experiments such as automatic MS^3 (MS/MS/MS) and MS^4 methods available on new instruments like Orbitrap Fusion Lumos, can be configured to automatically select and fragment ions from oxidized fatty acyl chains, providing valuable information for the assignment of exact oxidation modification sites.

Potentially, the ion mobility mass spectrometry (IMS) technique, capable of separating gas phase ions based on their molecular shapes (collisional cross-sections, CCS) as well as *m/z*, can be optimized to

resolve isomeric oxidized lipids prior to CID fragmentation by adding an additional layer of separation and reducing complexity of MS/MS spectra for isomers co-eluting in LC [69]. IMS-MS coupling already proved to be efficient in separation of lipid acyl-chain regioisomers and double bond positional isomers [69–71] as well as carbohydrate anomers [72]. These kinds of techniques are expected to be extended to the field of oxidized lipidomics.

To optimize the use of these new techniques for the identification of oxidized lipids, new software tools able to deal with the new information gathered using MS^n or IMS are required. For MS^n data processing, vendor specific programs have started to provide solutions for the unmodified lipid identification; for example, Lipid Search (version 4.1) can distinguish multiple TG isomers using MS^n data from Orbitrap Fusion Lumos Tribid mass spectrometer [73]. Further development and optimization are required for the identification of oxidized lipids. Third party vendor non-specific software that supports MS^n datasets e.g. MZmine2 [74] provides a framework to integrate new algorithms or extend existed algorithms for oxidized lipids driven by the community. For ion mobility, there are existing tools for the prediction of CCS values, e.g. LipidCCS [75]. However, the various forms and combinations of oxidative modifications will probably influence CCS value to a different extent. Thus, the transformation and evaluation of oxidized lipids has to be performed using standards including positional isomers.

4.2. Retention time prediction

Retention time prediction for oxidized lipids could offer an additional dimension for the assignment of oxidized structures to experimentally identified features. The retention time prediction is still scarcely used in software for LC-MS-based lipidomics, although retention time is a compound-specific property. The reason is that retention time prediction is far from being a trivial issue, strongly depending on the experimental condition used.

Recently, few proteomics applications have been reported to use machine learning techniques, which have been proven powerful in many research areas to make intelligent decisions, to assist peptide identification from MS/MS spectra [76,77]. Machine learning was also used for the RT prediction of some unoxidized lipids in reverse phase chromatography [78]. Descriptors used for modelling included molecular mass, logP, and calculated properties like surface area, electrostatic interactions, hydrophobic and hydrophilic effects, and polarization properties. When the model includes structures from different lipid classes (high structure variability) machine learning or QSPR models can offer robust tools for RT prediction. Unfortunately, RT prediction within a single lipid class (low structure variability) can be rather challenging, as recently described for steroids in reverse phase chromatography, due to the high number of isotopomeric structures for this lipid class [79]. Similarly, retention time prediction of oxidized species is complex, as different positional isomers are expected not to show significant differences in calculated polarity or hydrophobic/lipophilic balance, but *cis/trans* conversion of double bonds and potential intramolecular hydrogen bonds could increase the modelling complexity, by acting on the three-dimensional structure of the oxidized lipid. But how to select the best descriptors for modelling RT of oxidized lipids? Again, more experimental data are needed to generate a training set. Indeed, the major difficulties to train deep learning models for oxidized lipids include the shortage of reliable assigned spectra for data training, the lack of estimated oxidized lipid structure databases, and fast evaluation of computer assigned spectra during model training.

4.3. Increasing the coverage of oxidized lipid classes

The majority of published data on modified lipids describe species derived from oxidation of esterified PUFAs acyl chains (Fig. 1). However, in addition to oxygen addition and oxidative cleavage products, PUFAs can undergo other modifications including nitration and halogenation. Nitrated free fatty acids and more recently nitrated PLs and TAGs were identified *in vitro* and *in vivo*, and are usually associated with beneficial anti-inflammatory effects [80,81]. Detailed MS and MS/MS characterization was performed for several classes of nitrated lipids [82,83], however fragmentation rules observed in these studies were not implemented in identification software tools so far. Halogenated lipids represent another fraction of epilipidome. Thus, chlorinated PLs were proposed as pro-inflammatory markers formed upon activation of HOCl producing enzyme myeloperoxidase at the sites of acute inflammation [84–86]. Furthermore, nucleophilic amino groups at the head group moieties of aminophospholipids PE and PS provide another prominent modification site. Similar to the proteins (e.g. hemoglobin), aminoPLs undergo reaction with reducing sugars including glucose with a formation of Amadori products and further degradation to advances glycation end products (AGEs) know to be associated with development of hyperglycemia and diabetes [87–89]. Free radical modification of hydroxyl containing head groups of PLs were also proposed [90]. For all those modification types, a number of MS/MS specific fragment ions and neutral losses were described but not considered by high-throughput identification algorithms so far.

Currently, no software tool claims to support identification of modified lipids from all classes and multiple modification types. The major challenges come from the prediction of possible structures and corresponding fragmentation patterns. However, in future the enumeration of structures can be achieved by transferring the knowledge about the modification mechanisms to the computational algorithms optimized for each target lipid class and modification type.

4.4. Cross-platform nomenclature for oxidized lipids

The nomenclature proposed by the LIPID MAPS consortium for native unmodified lipids is used worldwide by the lipidomics community [1]. Furthermore, a shorthand notation system for lipid structures at different levels of structural confirmation was proposed by Liebisch et al. [91]. However, there is still no unified abbreviation system for oxidized lipids available to date. Historically, at the level of oxidized fatty acids abbreviations like 15-HETE or 12-HpETE for 15-hydroxyeicosatetraenoic and 12-hydroperoxyeicosatetrenoic acids are often used with minor variations when describing the R/S isomers. Prostaglandins can be named in slightly different ways usually sharing the common definition for ring types such as PGE_2 and E_2-IsoP. For oxPLs the majority of studies report partial identification reporting lipid class/species with the corresponding number of oxygens, e.g. PE(36:4) + 2O or 16:0/20:4(2O)-PE [7]. However, a clear and unambiguous nomenclature system for oxidized lipids capable of defining the exact structure including modification type, position, *cis-/trans-* and R/S isomers while still being flexible enough to match partial annotations is required to unify cross-platform data comparison and integration.

Recently, a few efforts have been made through the LIPID MAPS online tools and LPPtiger software. LPPtiger reports oxidized lipids at the level of modification number and type without specifying its exact position and stereochemistry. The LIPID MAPS online structure drawing tools (http://www.lipidmaps.org/tools/structuredrawing/StrDraw.pl) has implemented an abbreviation system for oxidized FA including a controlled vocabulary for 11 modification types (OH: hydroxy, NH2: amino, Me: methyl, Ke: keto/oxo, Ep: epoxy, SH: thio, My: methylene, Br: bromo, Cl: chloro, F: fluoro, CN: cyano) and has the capability to define the *cis-/trans-* and R/S isomers. This system can be extended to oxidized FA containing lipid classes such as PLs and TG. However, both available abbreviation systems have certain limitations. For example, the LPPtiger abbreviation use 1xKETO to represent a keto group instead of 1Ke which is shorter and simpler in the LIPID MAPS abbreviation. On the other hand, the LIPID MAPS abbreviation for lipids with terminal carboxylic acids use both OH and Ke at the same position, while LPPtiger indicates clearly the carboxylic acid using < C10@COOH > . Another issue is the optimization of shorthand notations and their

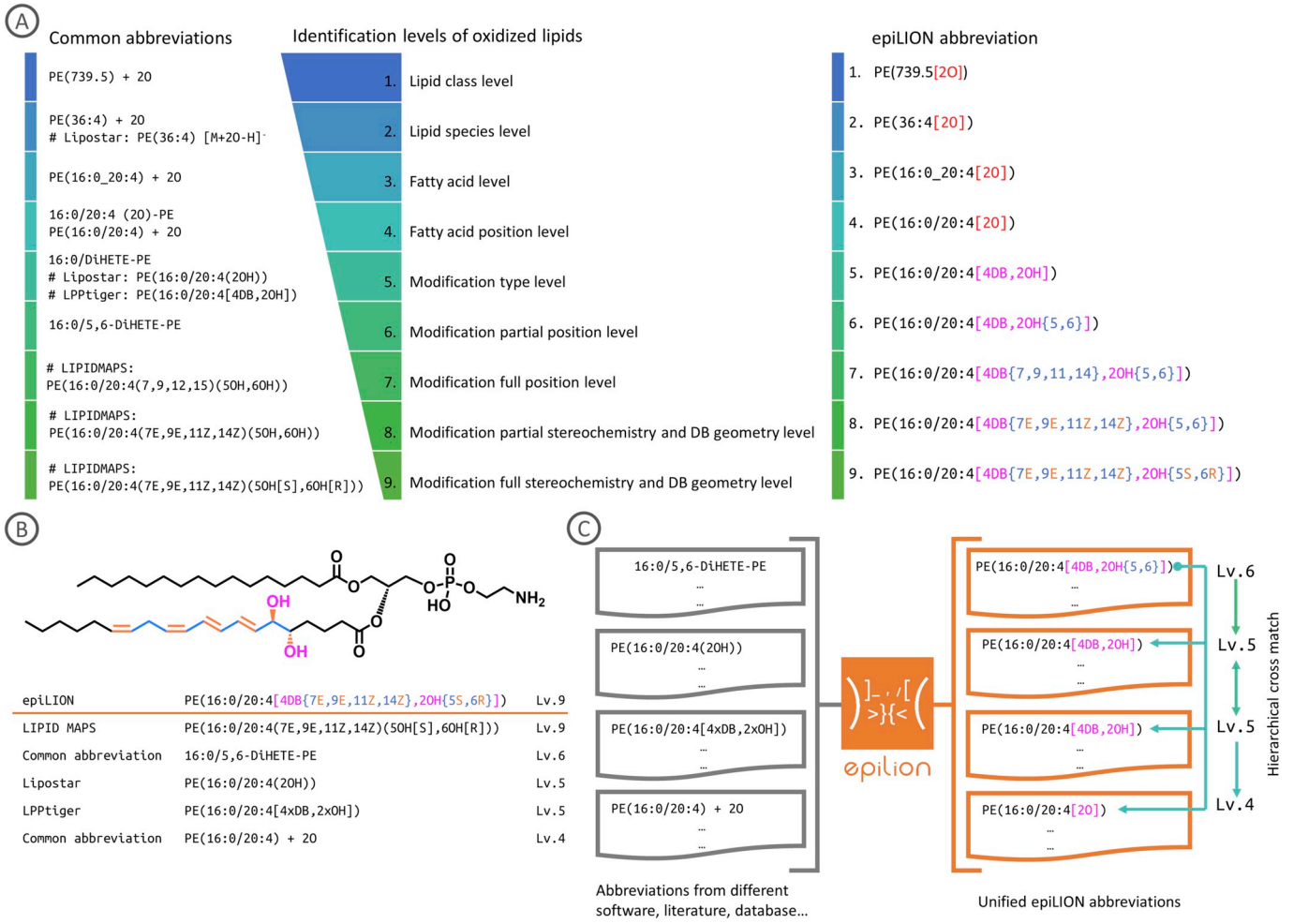

Fig. 4. Proposal for the epiLION (epiLipidome Identifier and Optimized Nomenclature) tool to provide unified abbreviations and ID conversion platform for oxidized lipids. (A) Overview of nine identification levels for oxPLs based on a specificity of structural assignment (middle) compared to the diversity of shorthand notations used by the community (left) and corresponding unified abbreviation system proposed by epiLION. (B) The epiLION abbreviation corresponding to 1-hexadecanoyl-2-(5S,6R-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphoethanolamine to unify common abbreviations as well as shorthand notations provided by LIPID MAPS, Lipostar and LPPtiger. (C) Conversion module available within epiLION to match different types of IDs reported in different studies, by different software and databases in unified epiLION ID supporting hierarchical cross-matching.

manual interpretation, for example in LIPID MAPS abbreviations for multiple OH groups it is needed to write “OH” each time, while LPPtiger has unnecessary “x” for multiplication and so on.

This lack of unified naming and abbreviation system and the possibility to map partial ID with more detailed levels of identification for oxidized lipids, makes the comparison of datasets derived from different laboratories and studies very challenging if at all possible. To close this gap, we propose the **epiLION** (**epi**Lipidome **I**dentifier and **O**ptimized **N**omenclature) tool to provide a unified abbreviation solution for both manually and software generated oxidized lipid IDs (Fig. 4). Furthermore, epiLION includes a conversion module which allows to covert different types of oxidized lipids identifications into unified and hierarchically structures abbreviations. A demo version of epiLION is available on Github (https://github.com/SysMedOs/epiLION). We believe that this proposal has the potential to unify the abbreviation system in epilipidomics and will finally allow high-throughput data comparison and integration.

4.5. Reducing computational costs

Another important aspect in developing efficient high-throughput software tools for redox lipidomics is their computational costs. From this perspective, the enormous structural diversity of oxidized lipids probably represents the most challenging tasks in developing dedicated software, as it is responsible for:

- Combinatorial explosion due to the multiple oxygenated species derived from a single lipid;
- Lack of knowledge about the number of possible oxidized structures effectively formed *in vivo* (risk of a high number of false positives).

As described above, the matching between experimental and *in silico* predicted fragment spectra is the most common approach for identification of oxidized lipids. However, the generation of a comprehensive database of all oxidized lipid species is extremely time and disk-space consuming, if not unfeasible, taking into account that true diversity of epilipidome is still an open question. For instance, in LPPtiger software, that provides a knowledge-based *in silico* oxidation algorithm, the prediction of oxPL structures and corresponding fragment spectra is still the most time-consuming step. Thus, novel strategies need to be developed in order to find high-throughput application. For instance, new improvements to optimize GPU-based parallel processing are currently under development.

Furthermore, combination of software tools able to provide a list of potential oxidized lipids in a high-throughput manner from “big data” acquired using modern LC-MS/MS lipidomics can be further used by a

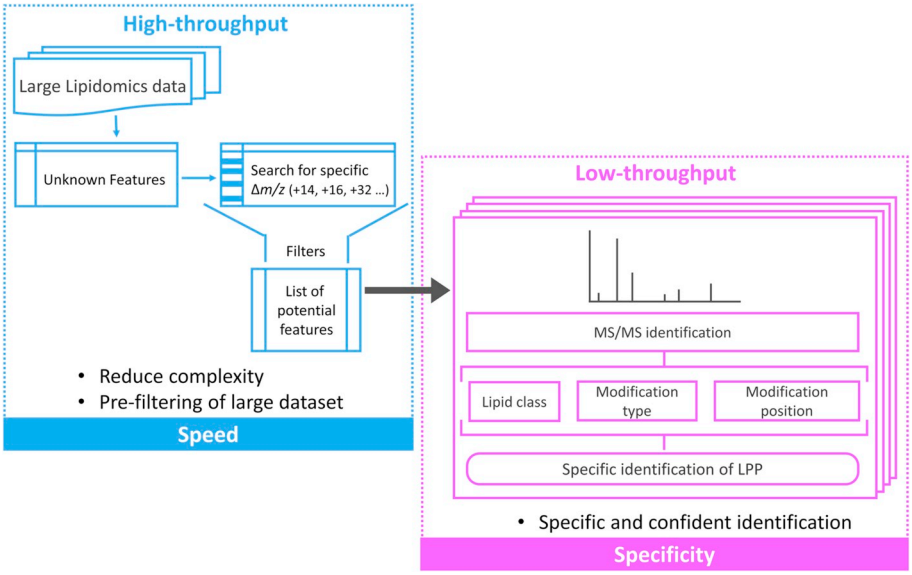

Fig. 5. Proposed optimized workflow for redox lipidomics by combining high-throughput screening for potential oxidized lipid features in large lipidomics datasets followed by specific identification of modification type and position using reduced dataset.

second software tool focused on the detailed specific identification of modified lipids including modification type and position specific isomers (Fig. 5). Such a complementary approach should significantly reduce the computational cost for mining potentially modified lipids in a large number of datasets at the first step and provide highly specific and accurate identification of oxidized species using a much smaller set of features during the second step.

5. Conclusions

Oxidized lipids display multiple biological activities and over the last decades were shown to be implemented in onset and progression of numerous human pathologies. Thus, high scientific interest in this field requires systems-wide analytical and bioinformatics solutions for high-throughput identification of the oxidized fraction of the epilipidome. A combination of modern analytical protocols based on LC separation coupled on-line to MS and MS/MS detection of modified lipids using an untargeted lipidomics workflow provides access to high-throughput omics datasets. However, identification of oxidized lipids remains a bottleneck of redox lipidomics. Although unified, fast, robust and specific software for high-throughput redox lipidomics is still missing, combined strategies might be applied to get closer to the goal.

Conflicts of interest

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.freeradbiomed.2019.04.027.

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