Published in final edited form as:

Biochim Biophys Acta. 2017 August ; 1862(8): 740–746. doi:10.1016/j.bbalip.2017.03.004.

Lipidomics: Prospects from a technological perspective

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Abstract

Over the last two decades, lipidomics has evolved into an ‘omics’ technology *pari passu* with benchmarking ‘omics’ technologies, such as genomics or proteomics. The driving force behind this development was a constant advance in mass spectrometry and related technologies. The aim of this opinion article is to give the interested reader a concise but still comprehensive overview about the technological state of the art in lipidomics, current challenges and perspectives for future development. As such, this article guides through the whole workflow of lipidomics, from sampling to data analysis.

Keywords

Lipidomics; Mass Spectrometry; Chromatography; Data processing; Lipid extraction; Biomarker discovery

1 Introduction

The term lipidomics was coined in 2003 by Spener et al. [1] and Han & Gross [2], and refers to the quantitative determination of all lipids in a given cellular system at a certain time point. Due to its sensitivity and specificity, mass spectrometry (MS) is the underlying technology of choice for simultaneous determination of large sets of lipids [3–5]. In its very beginnings in the nineties, even before the introduction of the term lipidomics, lipid MS was performed by direct infusion electrospray ionization (ESI) [6,7] which was termed ‘shotgun lipidomics’ later on [8]. In the following years, the analytical toolbox was significantly expanded by coupling of mass spectrometry with liquid chromatography (LC), thus increasing the selectivity and sensitivity of mass spectrometry based lipidomics, and resulting in the two major lipidomics setups nowadays: ‘shotgun lipidomics’ and ‘LC/MS lipidomics’ [9]. Due to an ever increasing number of chromatographic techniques, ionization methods, mass analyzers, vendors, and particularly combinations thereof, there is a huge

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Conflict of Interest

All authors declare no conflict of interest.

technological diversity even within these two categories. The aim of this article is to provide the inclined reader with critical evaluation of the trends in the field and identification of future potentials in lipidomics.

2 State of the Art

2.1 Sample preparation

The standard technique in lipidomic sample preparation is liquid-liquid extraction. Historically, the most common methods for total lipid extraction are based on biphasic chloroform-water mixtures, such as the methods established by Folch [10] and Bligh & Dyer [11] (Figure 1). While these methods are still widespread, lipidologists increasingly replace chloroform with other organic solvents, such as methyl-*tert*-butyl ether [12] for reasons of reduced toxicity and improved sample handling. Furthermore, biphasic systems are often replaced by simpler, monophasic precipitation methods to achieve the required sample throughput for large-scale lipidomic studies [13,14]. It should be noted however, that no single lipid extraction method is equally well-suitable for all lipid categories and classes [15]. Thus it is often beneficial to choose the extraction method depending on the analytes of interest and the exact research topic under investigation.

Solid-phase extraction (SPE) is usually employed in targeted analysis of lipids only. It offers an additional level of separation, and can be used for further fractionation of total lipid extracts [16] (Figure 1). This allows for either separation of phospholipids from other lipids [17], or separation of phospholipids or sphingolipids into individual classes [18,19]. Another benefit of SPE in lipidomics is the selective enrichment of low-abundance analytes of interest, such as long-chain base phosphates [20], steroids [21], or N-acylphosphatidylethanolamines [22].

2.2 Mass spectrometry and related analytical technologies

Shotgun lipidomics refrains from chromatography and therefore merely relies on mass spectrometry. Although alternative concepts, such as intra-source separation, or coupling to differential ion mobility spectrometry (IMS), have been developed [8,23], the basic criteria for selectivity are precursor masses of intact lipids and characteristic fragment masses. Unlike early instrumental setups, where direct infusion or flow injection coupled triple quadrupole systems were state of the art [6,7,24,25], nowadays many shotgun systems have evolved to high resolution instrumentation relying either on Orbitrap or on quadrupole time of flight (Q-TOF) technology [26–29], providing an additional gain in selectivity. Furthermore, the TriVersa NanoMate (Advion) enables a high degree of automation for sample infusion in conjunction with very high reproducibility, while the integrated nano electrospray ionization source increases sensitivity by at least two orders of magnitude [30].

Coupling of mass spectrometry to chromatographic separation adds additional selectivity by introducing retention time as a means of identification, thereby reducing complexity of the obtained mass spectra and increasing the peak capacity for each sample. Due to its applicability to a wide variety of biomolecules, ESI is the ideal ionization technique [31] in most LC/MS systems. In the last two decades, several LC/MS platforms have been

successfully used in lipidomic analysis, with LC triple quadrupole still being the most widely used system [31]. In such a setting, the instrument can be run either in a very targeted fashion by use of selected reaction monitoring (SRM) transitions for each lipid species [22,32–34], or in a semi targeted fashion using precursor ion or neutral loss scans on whole lipid classes [35,36]. In recent years, high mass resolution systems, predominantly based on either quadrupole time of flight (Q-TOF) [37–39], Orbitrap [40–42], or Fourier transform ion cyclotron resonance (FT-ICR) [43–45] mass spectrometers emerged as method of choice for LC/MS. The mass resolution in these systems ranges from 10,000 (Q-TOF) to over 1,000,000 (FT-ICR), providing increased analytical selectivity by elucidation of elemental compositions.

The main advantages and disadvantages of each instrumental setup are summarized in Table 1.

2.3 Data processing

There are several tools available for analysis of MS based lipidomics data (Table 2). The spectral similarity search tools LipidQA [46] and LipidSearch [41] comprise a spectral library of measured or *in silico* generated MS/MS spectra of lipid molecular species. The *in silico* spectra are typically based on observed fragmentation patterns derived from measured spectra. For spectral annotation, candidate reference spectra are first selected by filtering the database by the precursor m/z . Second, a similarity measure is calculated to express the resemblance of the measured spectrum to the reference spectrum. Third, potential lipid species, ranked by a score, are reported as result. LipidBlast [47] pursues the same strategy, however, this work is primarily focused on providing comprehensive spectral libraries, which may be utilized by available similarity search tools, such as the ones provided by the National Institute of Standards and Technology (NIST) [48] (<http://chemdata.nist.gov/mass-spc/ms-search/>).

In contrast to the usage of a rigid spectral library, the shotgun tool AMDMS-SL [49] utilizes an extensible ‘building block’ library. Each ‘building block’ represents a basic constituent inherent to the structure of most lipid species, i.e., backbone, head group, and aliphatic chains. This extensible concept is even further augmented by the molecular fragmentation query language (MFQL) of the LipidXplorer package [50]. MFQL is a flexible language for fragment definition, which allows for interconnecting several rules by logical operators. In most cases, the fragments of those queries represent the same structures as suggested by the building blocks of AMDMS-SL, however, they can be easily extended to any conceivable structures, ensuring utmost flexibility [51].

The software ALEX [52] provides a framework comprising several software packages for analyzing large amounts of shotgun data. Lipids are annotated on the lipid species level (no information about constituent fatty acyl chains), since the tool aims for robust high throughput data handling rather than obtaining structural details from MS/MS spectra. ALEX itself encompasses a lipid species database and several tools for processing high resolution Orbitrap data, including lock mass calibration. Notably, data are stored in table format, such as in relational databases, permitting the involvement of the auxiliary database

exploration tools Orange [53] and Tableau (<http://www.tableausoftware.com>) for further processing and data visualization.

For chromatography based approaches, the software LDA [54] provides means for peak deconvolution and quantitation. In lipidomics, overlaps of M+2 isotopic signals with the monoisotopic signals of lipid species featuring one double bond less occur quite frequently. The LDA's algorithm segregates such signals using a 3D-algorithm for confining peaks in time and m/z dimensions. Furthermore, false positive M+2 isotopic peaks are removed by verification of the isotopic patterns.

3 Perspectives and Limitations

The first and most important pitfalls in lipidomics occur already at sampling and subsequent sample processing. Biological samples, especially tissues, should always be flash-frozen in liquid nitrogen to stop enzymatic and chemical degradation processes. E.g., plasma levels of lysophosphatidylcholine and lysophosphatidic acid artificially increase when unprocessed samples are left to stand at room temperature [25,55], or when extraction is performed under highly acidic conditions [55], while on the other hand, elevated levels of monolysocardiolipin are suspected to originate from cardiolipin degradation during sample freezing [56]. When analyzing lysophospholipid regioisomers, one should keep in mind that they reach chemical equilibrium between their two forms in methanolic solutions above 20 °C, and at pH >6 [57]. Especially when potential degradation products of lipids, such as lysophospholipids, phosphatidic acid or oxidized phospholipids, are to be investigated, the entire sample preparation procedure must be rigorously evaluated to ensure that findings are not merely due to sample preparation artefacts. Generally, storing biological material before sample preparation or lipid extracts after sample preparation for excessively long periods of time is never beneficial, and samples should always be processed and analyzed as quickly as possible.

Standardization and nomenclature of lipids was lagging behind technological progress for quite some time. Only about a decade ago were lipids unified in a classification and nomenclature system [58], developed by leading experts from the US, Europe and Asia, with the LIPID MAPS consortium being the driving force. Based on this classification, the LIPID MAPS structure database is the most comprehensive compilation of lipid species up to date [59]. Additionally, the recently designed shorthand nomenclature for lipids [60] is an important amendment for standardization of the lipidomic language and for generation of databases. As depicted in Figure 2, mass spectrometry derived data of lipids can be acquired at different levels of structural detail, depending on instrumentation and techniques used. Therefore the proposed shorthand nomenclature reflects the ambiguities involved at every level of reporting, which gradually decrease as more elaborated methods stepwise uncover the molecular details of a lipid species. Only if a lipid is completely characterized down to the position and geometry of each double bond, the full LIPID MAPS name may be used. In all other cases, and this is in lipidomics rather the rule than the exception, the respective shorthand nomenclature shall be used to reflect the obtained structural evidence. In summary, the now unified nomenclature system is a prerequisite for reproducibility and exchange of data and paves the road for large research consortia.

A general bottleneck in lipidomics is the lack of pure reference compounds for validation of chromatographic and mass spectrometric characteristics, and as internal standards for quantitation. While a few hundred lipids are commercially available, for the vast majority of the speculated 100,000+ lipids in the biosphere, no reference compounds exist. This severely limits verification and quantitation of uncommon and/or minor compounds such as oxidized or bacterial lipids [40,61].

In recent years, the term biomarker search has found its way into lipidomics, which in this respect is often regarded as part of the umbrella term metabolomics [62–64]. Biomarker search in lipidomics is performed mostly in a non-targeted fashion and ideally with chromatographic coupling and the highest mass resolution possible [65–68] (Figure 3). While non-targeted methods open up vast m/z ranges for detection of as many compounds as possible, they often fail when it comes to discovery of low abundance compounds [69]. On the other hand, custom tailored targeted methods are more sensitive, but may miss unexpected lipids [69]. A solution to this Gordian knot can be the use of multiple platforms including different types of chromatography and ionization techniques, operated in a combination of non-targeted and targeted approaches. While providing the most comprehensive picture of a given sample, such kind of extensive analysis also requires a huge amount of available resources. An interesting and affordable compromise is described by Tarazona et al. [39], where the sample is split and subjected to both a targeted triple quadrupole method and a non-targeted high resolution method, each shedding light on the other's blind spots of detection. The biggest challenge in non-targeted analysis is compound identification by fragment mass spectra, the step from mere 'features' (m/z values with corresponding retention times) to meaningful chemical entities of biological relevance. While it is fairly easy to obtain database hits for bioactive molecules just by feeding in accurate m/z values for intact molecular ions, it is much harder to make chemical sense of fragment spectra. Unfortunately, fragment spectra are indispensable for identifying the correct chemical compound out of the plethora of possibilities featuring the same elemental composition. Although processing software has evolved in the last decade [41,47,50,54], automated interpretation of fragment mass spectra is still in its infancy. Performed manually, this task is up to date still the most time consuming and laborious step in any lipidomic biomarker search workflow, rendering any non-targeted biomarker search in lipidomics rather a matter of low than of high throughput. In contrast to genomics, transcriptomics, and proteomics, where high throughput identifications of biochemical entities can be produced by software routine in a much more standardized manner, lipidomics is currently rather a low-throughput discipline due to the required manual efforts, originating from chemical diversity. As a rule of thumb, while the few highly abundant major components in a sample are readily processed by algorithms at high identification certainty, the vast majority of low abundance components still require a lot of human intellectual input to achieve a sufficient level of certainty.

Contrary to completely non-targeted strategies, some groups meanwhile have reverted to targeted analysis to not get lost in the overwhelming wealth of data generated by non-targeted approaches. Such an approach requires a defined hypothesis, thereby narrowing down the analytical scope (Figure 3). Acidic lipids, such as (lyso)phosphatidic acid or sphingosine-1 phosphate, are easily missed by global approaches. Strategies for improved

detection of acidic lipids include specific acidic extraction protocols [70], custom tailored chromatography [71], or affinity based SPE [20]. Papan et al. recently proposed *in silico* combination of chemical building blocks for detection of new lipid species by shotgun lipidomics [72]. This approach nicely illustrates how non-targeted mass spectrometry settings can be usefully complemented with a target list generated at data processing by combination of building blocks. Derivatization is another strategy which, for the sake of increased selectivity and sensitivity, has experienced a revival in recent years [73–75]. While originally used exclusively in GC/MS, the benefits for LC/MS are twofold: Ionization efficiency may be enhanced by introduction of readily ionizing functional groups, and the addition of certain chemical moieties may result in increased selectivity by generation of characteristic fragment ions [76].

Last but not least, some recent technical advances are worth considering. The concept of supercritical fluid chromatography (SFC) has been around for a long time, but only within the last few years has SFC come to maturity due to improved coupling stability with MS. Better separation performance in less time at reduced costs for mobile phases are strong arguments for SFC to become *the* chromatographic separation technology for the future [77]. On the other hand, ion mobility spectroscopy (IMS) is promoted as a separation method complementary to chromatography, going even as far as completely replacing chromatography [23]. Although IMS shows promising potential for an additional layer of selectivity by introduction of drift times relying on compound specific collisional cross sections [78,79], it still suffers from a natural selectivity overlap with mass spectrometry (drift times usually correlate with molecular weight), which sometimes diminishes the chances of isobaric ions being separated by IMS. Moreover, IMS does not alleviate ion suppression effects in the ion source, because IMS separation is in any case located behind ion generation. Therefore, while IMS may add additional selectivity, it is far from being *the* solution for all the challenges unresolved by LC/MS. Consequently, it strongly depends on the analytical question if it is worthwhile to use such a device.

4 Conclusion

In summary, asking the right questions at the right place is more important than ever in lipidomics. A defined hypothesis, carefully planned sampling, sample storage, and sample preparation are still the basis for sustainable scientific progress, whereas the currently omnipresent possibility to generate huge amounts of data by mass spectrometry and related technologies is only use- and meaningful when these prerequisites are fulfilled. Only if this is the case will bioinformatics and biostatistics be able to reveal more than the tip of the iceberg from the data we have acquired.

Acknowledgements

This work was supported by the Austrian Science Fund (FWF): [P 26148].

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Highlights

- We review the current state of the art in the field of lipidomics.
- We describe sample preparation techniques and available instrumentation.
- We critically evaluate future perspectives and limitations of lipidomics.

Figure 1.
Overview of lipidomic sample preparation strategies.

Figure 2.

Different levels of lipid identification, exemplified by 1-palmitoyl,2-oleoyl-sn-glycero-3-phosphocholine. As the information about the lipid grows more comprehensive, the effort necessary for identification (example techniques right of the pyramid) multiplies. PC, phosphatidylcholine; PIS, product ion scan; HR/AM, high resolution accurate mass; MS², tandem mass spectrometry

Figure 3.

Targeted and non-targeted analysis in lipidomics. While targeted lipidomics approaches often result in accurate quantitative data of a limited number of lipids, the focus of non-targeted methods is set to recognition of specific regulation patterns and subsequent identification of its key compounds. DDA, data-dependent acquisition.

Table 1

Summary and characteristics of the most widely used instrumental setups in lipidomics. RP, reversed phase; HILIC, hydrophilic interaction liquid chromatography; NP, normal phase; SFC, supercritical fluid chromatography; FT-ICR, Fourier transform ion cyclotron resonance; TOF, time of flight, ECN, equivalent carbon number.

	pro	con
Shotgun (NanoMate, flow injection)	Constant ion suppression effects	No separation of isobaric lipids in MS! Data handling difficult, requires specialized software packages
Chromatographic approaches	RP	Molecular species of the same lipid class may ionize differently due to a different chemical environment at different retention times, resulting in variable intensities, ion suppression effects not constant for all species of one lipid class
	HILIC / NP	Isotope correction algorithms required
	SFC	Not very widespread Reproducibility issues are unknown up to date, due to the novelty of the method
Mass spectrometer types	Triple quadrupole (tandem in space)	Offers only unit resolution SRM methods limited to predefined analytes, no way to re-analyze data
	Ion trap (tandem in time)	Suffers from low mass cutoff Offers only unit resolution
	Orbitrap / FT-ICR	Expensive Slow duty cycle when operated at full resolution capacity
	TOF	Narrow linear range Sensitivity often suffers with increasing resolution

con

pro

Table 2

Overview of selected lipidomics MS analysis tools. “Quantitation” indicates whether the software reports quantitative values. “Structure” specifies if the tool reports results based on the lipid molecular species level, i.e., providing information of constituent chains and/or *sn*-positions. “Customizable” stands for customization options for self-defined lipid species and potential extensibility.

Name	Type	Quantitation	Structure	Customizable	Availability	URL
ALEX	shotgun	yes	no	yes	free	http://www.msLipidomics.info
AMDMS-SL	shotgun	yes	yes	yes	free	https://pharmacometabolomics.duhs.duke.edu/resources-tools/sanfordburnham-medical-research-institute
LDA	LC	yes	no	yes	free	http://genome.tugraz.at/lda
LipidBlast	both	no	yes	yes	open-source	http://fiehnlab.ucdavis.edu/projects/LipidBlast/
LipidQA	shotgun	yes	yes	no	free	http://lipidqa.dom.wustl.edu/
Lipid Search	LC	yes	yes	no	commercial	marketed by Thermo Fisher Scientific
LipidXplorer	shotgun	yes	yes	yes	open-source	https://sourceforge.net/projects/lipidexplorer