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Opinion article

Green light for lipid fingerprinting

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ABSTRACT

The use of targeted lipidomic approaches for the analysis of plant lipids has steadily increased during recent years. We review recent developments of these methods and suggest the introduction of discovery lipidomics as additional approach through a new workflow, lipid fingerprinting, that integrates the advantages of shotgun lipidomics (quantitative data) with LC-MS-based strategies (higher resolution and/or coverage). This article is part of a Special Issue entitled:BBALIP_Lipidomics Opinion Articles edited by Sepp Kohlwein.

1. Introduction

From the early days of chromatography, when Tswett first separated leaf pigments [1], lipid analysis has been always connected with analytical developments. The continued development of analytical tools and techniques has defined the discipline of lipidomics, focusing on improvements mainly in mass spectrometry and in separation techniques [2,3]. Specifically, these improvements have addressed two key analytical challenges: naturally occurring lipid signals are typically found in relatively narrow mass ranges and can often suffer from isobaric interferences; and lipids present in high concentration partially or fully suppress the detection of numerous important low abundance lipids. The resolution of these two issues has been addressed in recent years by a number of technological analytical advances. However, further improvements are now often accompanied by compromise, namely that growing the number of detected lipid molecules often reduces the number of analyzed samples per time. As investigators attempt to capture the huge array of lipids typically found in biological samples with ever greater accuracy, improvements are made in sample preparation, chromatographic separation and mass spectrometric detection, but this is always at the expense of measurement time and costs. It is possible that developments may allow the capture of many hundreds of different plant lipid molecular species across multiple classes in a single analytical run. Here, we present our thoughts on the opportunities for discovery lipidomics provides and a new workflow, lipid fingerprinting, that integrates the advantages of shotgun lipidomics (quantitative data) with LC-MS-based strategies (higher resolution and/or coverage).

2. The challenges of lipidomics

2.1. Lipid extraction

Prior to any discussion of lipid analytical measurement, sample processing and extraction must first be addressed. Effective improvements in lipidomic analysis begin with sample extraction; the choice of methodology defines the range of metabolites available for analysis and the likely throughput. Traditionally, isolation of lipids has relied on general extraction procedures established by Folch et al. [4] and Bligh and Dyer [5], with frequent modifications for specific tissues, scaling of volumes and matrices. However, these methods typically encounter problems when adapting them to automated liquid handling systems and processing large numbers of samples. The development of protocols using the less toxic methyl *tert*-butyl ether (MTBE) has now provided an alternative in which the low density lipid-containing organic phase forms the upper layer during phase separation [6]. The successful extraction of the main lipid classes using MTBE has enabled the development of well-plate based high-throughput analysis. Accurate and rapid lipid extraction using well plates has been the ambition of many in the lipidomic community. For example, an automated chloroform-free method was developed by Lofgren et al. [7,8] for the 96-well extraction of plasma lipids. With compatible recovery of the main lipid classes to the Folch method, this BUME (butanol/methanol) method enables the effective processing of large sample numbers. Following the

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same line, a single phase method based on isopropanol, hexane and water was developed for plant tissues [9]. However, even with these developments extracts contain complex mixtures of metabolites which have the capacity to interfere with accurate mass spectrometry (MS) based analysis. Therefore, when trying to undertake the in-depth characterization of lipid classes, further clean-up is often required, involving either liquid/liquid partitioning or solid-phase extraction. The issue of lipid stability must also be considered within any extraction approach; specific lipid species are more likely than others to undergo degradation during the extraction process. For example, oxylipins require specific extraction methodologies to retain their original metabolic form [10]. Overall, when selecting any lipidomic analytical strategy, the number and type of operations necessary for lipid extraction must be considered.

2.2. Analysis using mass spectrometry

In terms of mass spectrometry detection, a major milestone in the field of lipid analysis was the development of electrospray ionization (ESI) and its connection to tandem mass spectrometry (MS/MS) in the field of mammalian lipids [11,12]. Cell extracts are analyzed by direct infusion MS using precursor ion scans (PIS) and neutral loss scans (NL) to identify key lipid fragments. The relative simplicity of direct infusion ESI-MS/MS, fast analysis, and flexibility to detect various lipid classes within a single run has resulted in the widespread adoption of this approach. In its current form, ESI-MS/MS methods now widely use automated nanospray techniques. The use of NanoESI-MS/MS has extended the lipid analysis of low volume samples that would otherwise not be practical with other approaches. The transition of ESI-MS/MS methods to plants, which have a much more complex matrix took a further five more years [13]. Since these methods relied exclusively on a simple extraction procedure and direct mass analysis for lipid identification, they increased dramatically the speed and coverage of analyzed lipid samples at relatively moderate costs. Complimenting traditional lipid analytical approaches, the establishment of direct infusion ESI-MS/MS for the characterization of plant lipids enabled the comparison of the lipid profiles from plants subjected to forward- or reverse-genetic manipulation, developmental and physiological phenotyping, and unraveled metabolic bottlenecks associated with molecular engineering. However, it is apparent that methods using tandem MS in a class-specific or targeted way are restricted in their potential to detect and identify unknowns [11]. Furthermore, during recent years it became clear that direct infusion methods suffer from ion suppression. Not all molecular species are identified with equal efficiency, in particular acyl chain length, degree of unsaturation and lipid concentration all impact on the instrument response. Indeed in plant lipidomics, which covers a huge diversity of starting material including crops, microalgae, roots, shoots and seeds, matrix interference and ion suppression can make the analysis of low abundant species extremely difficult [14]. In addition, even if with increasing mass resolution, the high number of possible isobaric ions, which may occur in lipid extracts, limits the selectivity of the direct infusion approach [15]. In the search to characterize the lipidome at a greater depth, it has been observed that isobaric interference can be often neglected for major species, whereas for minor species, fragment ion spectra often remain unclear and false positives may occur [16]. However, it should be pointed out that a major strength of all direct infusion or so-called shotgun approaches, besides their high speed and comparatively low costs, is that at least semi-quantitative data can be obtained by adding just a reasonable number of isotopically labelled standards.

2.3. The development of analytical approaches for lipidomics

As discussed, the enormous diversity of lipids in biological extracts has created an ongoing analytical challenge that often requires the adoption of new strategies for the deconvolution of complex isobaric ions. In particular, ion mobility-mass spectrometry (IM-MS) in which ions are separated by their size, shape, charge and mass depending on different mobility in low or high electric fields, has provided a new dimension in the analytical workflow. The use of IM-MS specifically allows for the separation of isobaric species, reduction of background noise and increased selectivity via the separation of interfering metabolites from target lipids. Moreover, IM-MS provides a unique descriptor of the physiological properties of a compound (collision cross-section value; CCS) based on the time required for any given ion to cross the chamber. CCS values provide another means of compound annotation and the benefits of IM-MS in lipidomics have been demonstrated, for example, differences in drift time have been used to separate phospholipids with different degrees of unsaturation, linkage type and head-group [17,18]. Research has now established ion mobility as a method that provides a separate and complimentary approach to increasing selectivity, enhancing the detection of low abundance species [19]. Indeed, it also has the potential to reduce the number of steps required for successful sample extraction and clean-up. However, any issues involving ion suppression at the ion source will still remain, limiting the utility of IM-MS for the quantification of low abundant ions in complex biological samples.

Often not represented in any total extraction of lipids is the spatial distribution or compartmentation of lipid species in tissues or samples. The development of MS-imaging has, particularly in plant lipidomics, begun to address this lack of spatial information. Matrix-assisted laser desorption ionization (MALDI) coupled to high-resolution mass spectrometry (MS) has emerged as a new tool for the localization of lipid metabolites directly in plant tissues with 5-to-50 μm spatial resolution [20,21]. Imaging the location of phosphatidylcholines (PCs) and triacylglycerols (TAGs) in seeds by MALDI-MS not only demonstrated the compartmentalisation of lipid metabolism but also the metabolic precursor-product relationships of seed oil biosynthesis [22].

Therefore, even with the technological advances described above, the analysis of (mainly) minor lipids such as lipid mediators has required attention and has led to the development of numerous specific liquid chromatographic separations prior to mass analysis [10,23–26]. Primarily these methods have included hydrophilic interaction chromatography (HILIC) and reversed phase (RP) chromatography. The former is very well suited for lipid class discovery [27], but is limited in resolving lipids within the same class [28]. However, this drawback can be overcome by combining it with RP methods that allow the separation of molecular species including isomeric pairs [29] and will most likely lead to the development of two dimensional LC-MS methods in the near future. In any case, when relying on a single chromatographic run both HILIC and RP methods usually focus on certain lipid classes for optimizing separation and thereby increasing the number of detected species, but this may compromise the analysis of other lipid classes. Only recently, a method for plant lipidomics has been developed that increased the coverage of measured species at a reasonable time by covering a great number of lipid classes [9]. This approach (Fig. 1) used an organism-specific database, created from a system-wide analysis, to generate a targeted list of candidate multiple reaction monitoring (MRM) transitions. Such an approach provided a high selective and sensitive method for the analysis of 393 molecular lipid species in leaves from the model plant *Arabidopsis thaliana*. However, it should be noted, that almost all lipidomic methods that rely on the combination of liquid chromatography with mass spectrometry (LC-MS) suffer as quantitative methods. Since the ionizing conditions of lipid molecules at different retention times within a gradient profile may differ remarkably in LC-MS-derived methods; typically, they would require a much larger number of internal standards that are often not available. Therefore, the main challenge for lipidomics still remains the development of a single-method with a broad-coverage of almost all lipid classes and abundances, whilst retaining the selectivity, sensitivity and sample throughput of methods that focus traditionally on much smaller groups of lipids.