Technical Overview

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Lipidomics Analysis with Lipid Annotator and Mass Profiler Professional

Introduction

Lipidomics is the comprehensive and quantitative measurement of lipids present in an organism. Lipids are key to cell membrane function, energy storage, and cell signaling. To understand the lipidome, it is desirable to characterize all lipids present. However, the diverse chemical nature and large number of possible lipids creates a substantial analytical challenge. Shotgun lipidomics is a well established infusion-based technique designed to rapidly yield quantitative lipid class data on a small number of lipids using class internal standards. This approach does not provide unambiguous identification of the lipids, as lipid isomer information is lost. Another significant limitation of shotgun lipidomics is ion suppression of some lipid classes caused by the differences in ionization efficiencies.

Profiling lipidomics is a more comprehensive approach, yielding relative quantitation and identification of hundreds of lipids, including isomers, in a single analysis. This analysis is facilitated by advances in chromatography such as supercritical fluid chromatography and 2D-LC, as well as the development of ion mobility mass spectrometry. Lipid profiling allows separation of lipid isomers and isobaric lipids; however, lipid identification by mass spectrometry is still a significant challenge. Complete lipid identification includes class, elemental composition, R group size and location, number and location of double bonds, and double bond orientation (*cis/trans*). While there are small MS/MS spectral libraries from authentic standards for identification, most lipid MS/MS spectra are annotated using *in silico* MS/MS libraries.

The inherent complexity of lipidomics data sets requires advanced software tools for data analysis. This Technical Overview introduces lipid-specific software for the Agilent LC/MS profiling lipidomics workflow.

Overview of the Agilent LC/MS lipidomics workflow

Sample preparation and the chromatographic method impact the classes of lipids resolved and detected. The Agilent lipidomics workflow requires the use of a high-resolution LC/Q-TOF or IM Q-TOF mass spectrometer for data acquisition. To provide the highest-quality quantitative data, samples are analyzed in MS1 mode. In the same worklist, MS/MS data are acquired, typically on a pooled sample, and may use iterative MS/MS acquisition where repeat injections of the sample improve MS/MS coverage.[1] Agilent MassHunter Lipid Annotator software is then used to annotate lipid MS/MS data, and convert those results into an accurate mass, retention time, and CCS (IM workflow) database. This lipid profiling approach is based on a stable chromatographic separation, as retention time is required when the lipid database is used to perform either targeted extraction of annotated lipids, or to annotate differential features from an untargeted discovery experiment (Figure 1). The targeted data mining approach provides more efficient and consistent results for large lipidomics projects. The untargeted approach, which can be performed on the same MS1 dataset, provides a more complete view of all lipids present in the sample set. Targeted or untargeted feature extraction results can be imported into Agilent MassHunter Mass Profiler Professional (MPP) software for statistical analysis.

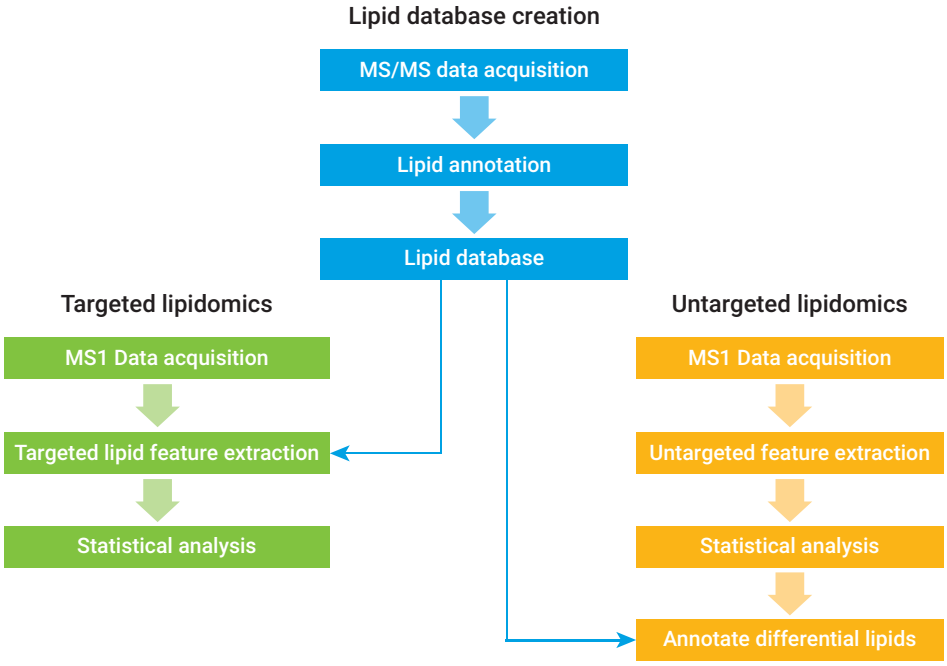

Figure 1. The Agilent MassHunter Lipid Annotator software generates a database (PCDL), which can be used in either a targeted or untargeted workflow.

MassHunter Lipid Annotator

Confident lipid annotation uses MS/MS spectra to match product ions against *in silico*-generated databases. There are two key factors in successfully annotating lipids: a high quality, comprehensive spectral database, and a well designed algorithm for matching acquired MS/MS spectra to theoretical lipid spectra. For *in silico* spectral matching, Agilent provides MassHunter Lipid Annotator software, which uses an algorithm that combines probability density, Bayesian scoring, and a nonnegative least square fit to search a theoretical lipid library.

In the Lipid Annotator algorithm, the mass (Score (Mass)), isotope pattern (Score (Isotop.)), and MS/MS spectra (Score (Frag.)) of a given feature are each considered independently of one another, and multiplicatively contribute to the probability density (Score (Total)) of a possible lipid match. Probability density is also invoked when evaluating an individual MS/MS spectrum, as both the product ions matched, and the impurity product ions are considered independently of one another, and multiplicatively contribute to the Match Score. Note that the Match Score represents only the MS/MS spectrum shown in the mirror plot, whereas the Score (Frag.)) combines all the MS/MS spectra information in the data file.

Lipids present an additional challenge in that often a mass, isotope pattern, and even MS/MS spectra could potentially be explained by many lipid annotations. If the possible annotations belong to different lipid classes, then Bayesian probability logic is used to determine the top candidate (for example, PE versus PC). This is the probability (Probab.) reported in the top table in the Match Details view. If the possible annotations belong to the same lipid class, the algorithm uses a nonnegative least square fit to determine the abundance percent rank for the different lipid constituents (for example, PC 16:1_16:1 versus PC 14:0_18:2) that may be present in a sample, and to choose the most dominant lipid constituent. When a dominant constituent cannot be discerned, only the sum composition will be annotated in the database. The *in silico* library is a modified version of LipidBlast, that was developed initially by Kind *et al.*, then further improved by Tsugawa *et al.*[2,3] The database, which covers mammalian and some plant and bacterial lipids, contains 58 unique lipid types when considering all ether and oxidized lipids as single lipid types. In addition, the 14 SPLASH Lipidomix Standards (Avanti p/n 330707) were added to the library to enable lipid class normalization. Lipid Annotator takes special care not to overannotate lipid entities by only providing the level of structural information confidently informed by the MS/MS spectra.

A Lipid Annotator project can contain multiple MS/MS data files to be processed together as a batch. The project data files must use the same chromatography, and be from the same sample origin. Lipid Annotator extracts features that are defined by molecular weight, retention time, *m/z*, and abundance. The associated isotopes and MS/MS spectrum for each feature are also extracted from the data. The results from this data processing can be viewed in the Feature or Match Detail view. The Feature view (Figure 2) allows the user to get an overview of the results. The Feature table contains all features with associated MS/MS spectra found in the project. There is a feature plot where results can be displayed as abundance versus retention time, abundance versus *m/z* or *m/z* versus retention time. This allows for a quality assessment of the annotation results. The pie chart shows the percent of lipids annotated for each lipid class.

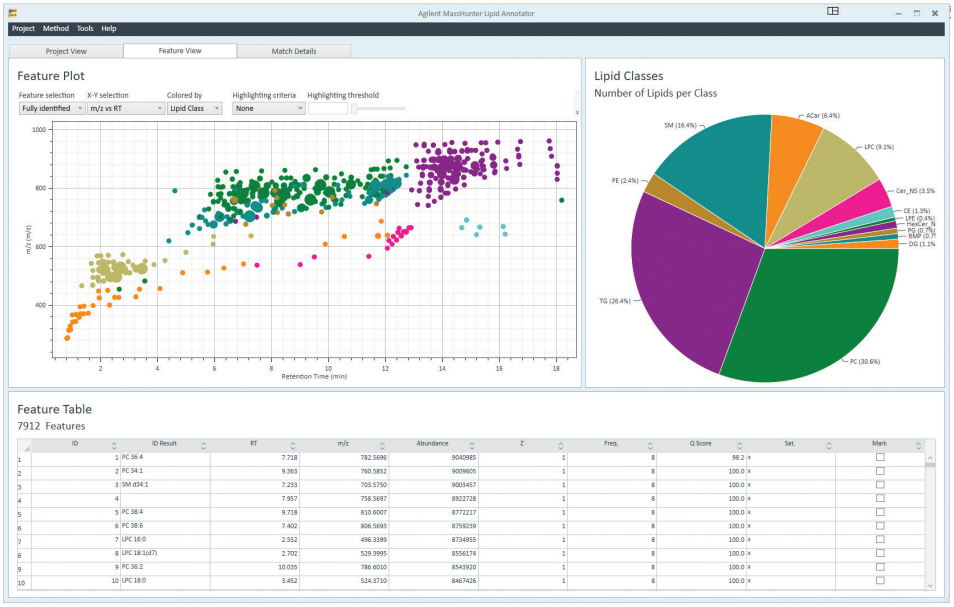

Figure 2. Feature view provides a scatterplot, pie chart, and feature table to provide an overview of the annotation results.

The Match Details tab (Figure 3) provides a more detailed view for examining each lipid match. The Lipid Sum Composition table contains information for each annotated lipid. Sum composition includes the lipid class, total number of carbons, and total number of double bonds. The Lipid Constituent table provides possible R group matches for a given sum composition. The Lipid Constituent table includes the abundance percent score for each possible constituent, and if one constituent can clearly be distinguished, it will be reported as the dominant constituent. The mirror plot shows the observed MS/MS spectra on the top (red) and the *in silico* database MS/MS spectra on the bottom (blue) for the selected lipid constituent.

The Lipid Annotator project results can be exported into an Agilent database file (PCDL format) or as a .csv file. The resulting PCDL database can be examined and edited in Agilent PCDL Manager. This database can be used for targeted feature extraction in MassHunter Profinder. In an untargeted discovery experiment, the user-created database can be used to annotate features using Agilent ID Browser.

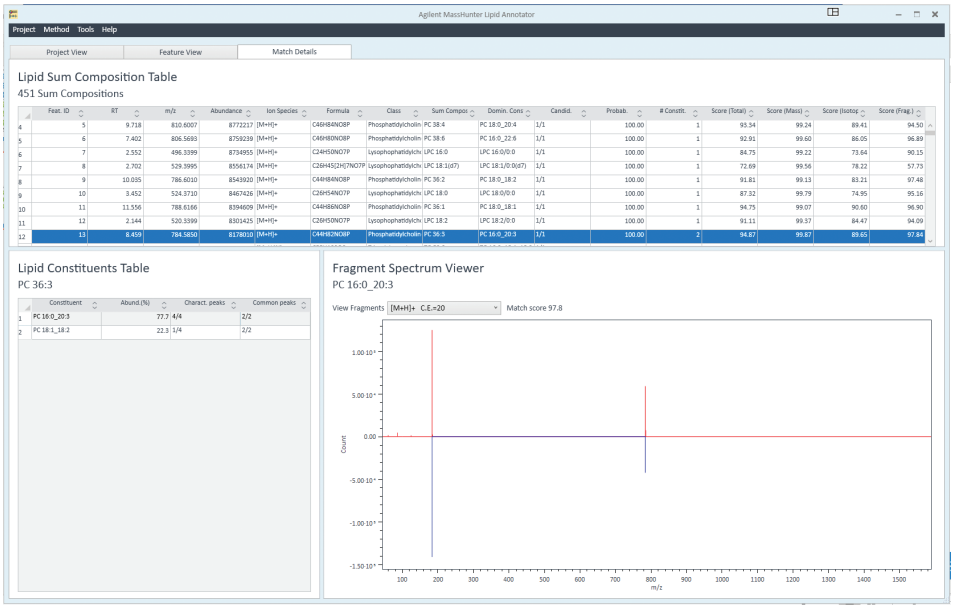

Figure 3. Match details provides a table of lipid annotated features, a lipid constituent table, and a mirror plot to allow the user to investigate the lipid matches.

MassHunter Mass Profiler Professional

Agilent offers advanced statistical analysis software, MPP, for examining and interpreting complex lipidomics results. To enable annotated lipid analysis, a lipidomics experiment type has been added to MPP. The lipidomics experiment type supports lipid class-based internal standard normalization for greater relative quantitative accuracy. It also has several visualizations to support lipid analysis, including a lipid matrix plot and a Kendrick mass defect plot.

Lipid classes are based on common chemical structures that have similar properties, thus class-based normalization is an effective strategy to mitigate variations across and within sample batches. The addition of standards (such as SPLASH Standards) to samples prior to lipid extraction can be used to normalize differences in extraction efficiencies as well as instrument response. MPP recognizes the lipid class from the annotation, and can optionally apply specified class standards for normalization.

Lipid class information is also used in some of the special visualizations supported for lipidomics in MPP. Lipid matrix plots are MPP visualizations that show changes in annotated lipid abundances. Three types of lipid matrix plots provide the ability to view lipid abundances between lipid classes, within lipid classes, and at the sum composition level. Figure 4 is an example of a matrix plot for the lipid classes across plasma samples.

Another lipidomics visualization is the Kendrick mass defect plot. The aliphatic nature of lipid classes results in visible trends when lipids are plotted by mass defect versus nominal mass. The Kendrick plot enables the detection of subtle differences in lipid structures such as the presence of a double bond. This plot can be particularly useful in the untargeted workflow when speculating on the putative identity of an unannotated lipid feature (Figure 5).

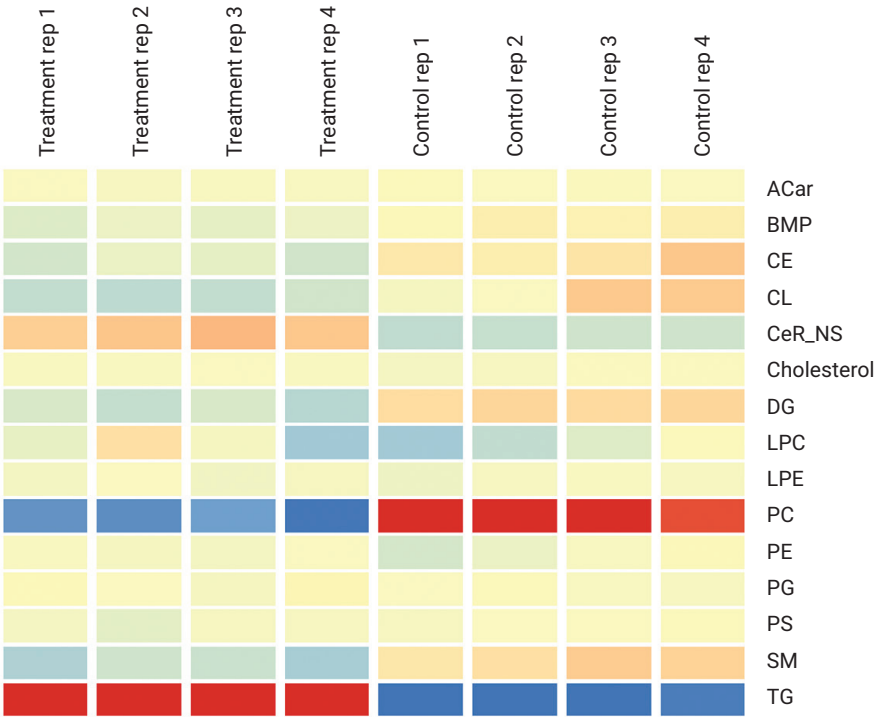

Figure 4. MPP lipid matrix plot by lipid class. The normalized abundances for all annotated lipids within a lipid class are summed (rows) and plotted versus the individual samples (columns).

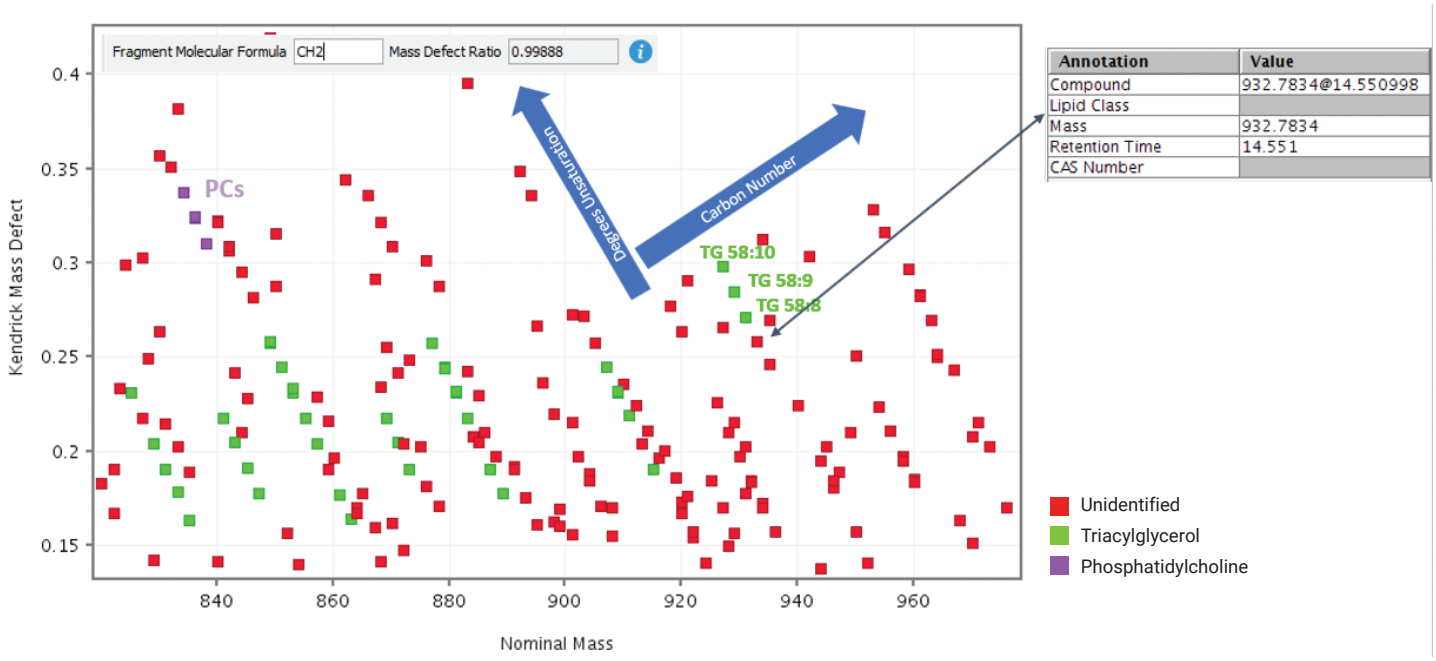

Figure 5. View of MPP Kendrick mass defect plot. Trends in carbon number and degree of unsaturated bond content are indicated with blue arrows. Annotated triacylglycerol (TG) lipids are here shown in green, and unidentified compounds in red. A statistically significant unidentified compound (indicated with an arrow) is near a group of annotated triacylglycerol (TG) compounds. Further inspection with the entity inspector shows a mass that has a 0.63 ppm difference from TG 58:7.

Conclusion

The Agilent lipid analysis workflow supports LC/Q-TOF and IM Q-TOF instruments, and includes software that enables in-depth analysis of lipids in large sample sets. Lipid Annotator software enables quick creation of a user-specific lipid database for rapid, consistent targeted analysis of large lipidomics datasets. The lipidomics experiment type in MPP is designed to support the statistical analysis of lipid results. This comprehensive workflow supports targeted and untargeted profiling lipidomics to easily explore changes in lipid profiles between experimental groups.

For more information about the Agilent LC/MS lipidomics workflow, visit: https://www.agilent.com/en/products/software-informatics/masshunter-suite/masshunter-for-life-science-research/mass-profiler-professional-software.

References

1. Iterative MS/MS Data Acquisition Combined with Lipid Annotator Software Improves Coverage of the Plasma Lipidome Using 6546 LC/Q-TOF. *Agilent Technologies Application Note*, publication number 5991-0775, **2019**.
2. Kind, T. *et al.* LipidBlast *in silico* tandem mass spectrometry database for lipid identification. *Nature Methods* **2013**, *10(8)*, 755–758.
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