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Uncovering More Biological Insights in Your Samples with Routine LC/Q-TOF Workflows for Metabolites and Lipids

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Introduction

Overcoming Untargeted Metabolomics and Lipidomics Challenges with New LC/Q-TOF, Software Tools, and Supportable Methods

Untargeted Omics analysis is still a preferred technique for discovery biology researchers. Unfortunately, this technique is hindered by lack of standardized methods, software tools that limit the building of MS/MS and Retention Time (RT) libraries as well as efficient extraction, statistical and identification software needed for unknown workflows.

Described here is a workflow extracting lipids and metabolites from the same mouse plasma aliquot, collecting the data with an Infinity II Bio LC and a Revident LC/Q-TOF (Figure 1) and analyzing the data sets with unique software tools and new statistical software, MassHunter Explorer.

Each step and method in this workflow has been standardized allowing for these methods to be easily transferred to other systems. This standardization also enables the support and use of spectral and RT databases curated by Agilent on these LC methods. This gives researchers the fastest way to implement the workflow shown here with the most confidence in their results.

Figure 1. Standardized LC configuration used a 1290 Infinity II Bio LC (left) which is metal free to give best peak shape of metal sensitive analytes. Coupled to it is a Revident LC/Q-TOF (right) which provides robust and reliable small molecule analysis. Not shown is a Metabolomics Bravo platform for sample preparation.

Experimental

Lipids and Metabolites are Extracted from the Same Plasma Sample to Gain More Information

Twenty female and twenty male biological replicate mouse plasma samples were processed on the Metabolomics Bravo platform with previous protocols.[1] The samples underwent extraction using the Captiva EMR-Lipid plates where the first set of solvents captures the lipids and elutes the metabolites, and the second set of solvents releases the lipids into a separate collection plate. This allows dual analysis of metabolites and lipids from the same aliquot of sample, reducing experimental variability (Figure 2).

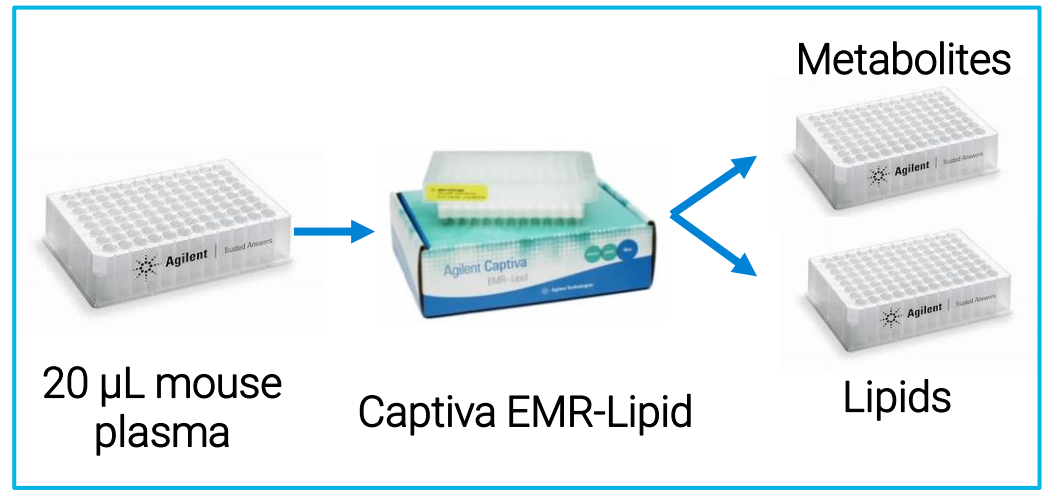

Figure 2. Sample prep workflow illustration. The samples were pipetted onto a 96 well plate and extracted using a Metabolomics Bravo platform (not shown) and the Captive EMR-Lipid SPE plates. The output are two separate collection plates each holding the metabolite and lipid fractions for LC/MS analysis.

The chromatographic methods for the lipid and metabolite methods are described thoroughly elsewhere and are supported in customer labs with SOPs for easy transfer.[2,3] Briefly, separation of metabolites happens in a 23 min method using a HILIC-Z 150 mm column and the lipids separate in 16 min with a Zorbax C18 100 mm column. These analyses can be carried out back-to-back on the Bio LC with a brief solvent wash between buffer systems with no negative impact on data quality.

The Revident LC/Q-TOF was tuned in *m/z* 1700 mode and operated in MS1 mode *m/z* 60-1000 for each individual sample. Positive and negative ion mode data was collected for metabolites and only positive mode for lipids. A pooled QC was made and injected throughout the MS1 worklist for data quality evaluation. Additionally, the pooled QC was used for an iterative MS/MS acquisition (n=8) which is analyzed in either Lipid Annotator 1.0 or MassHunter Qual 12.0 for custom library building (Figure 3). All analytes identified in the iterative workflow are organized with RTs using ChemVista spectral and RT library software.

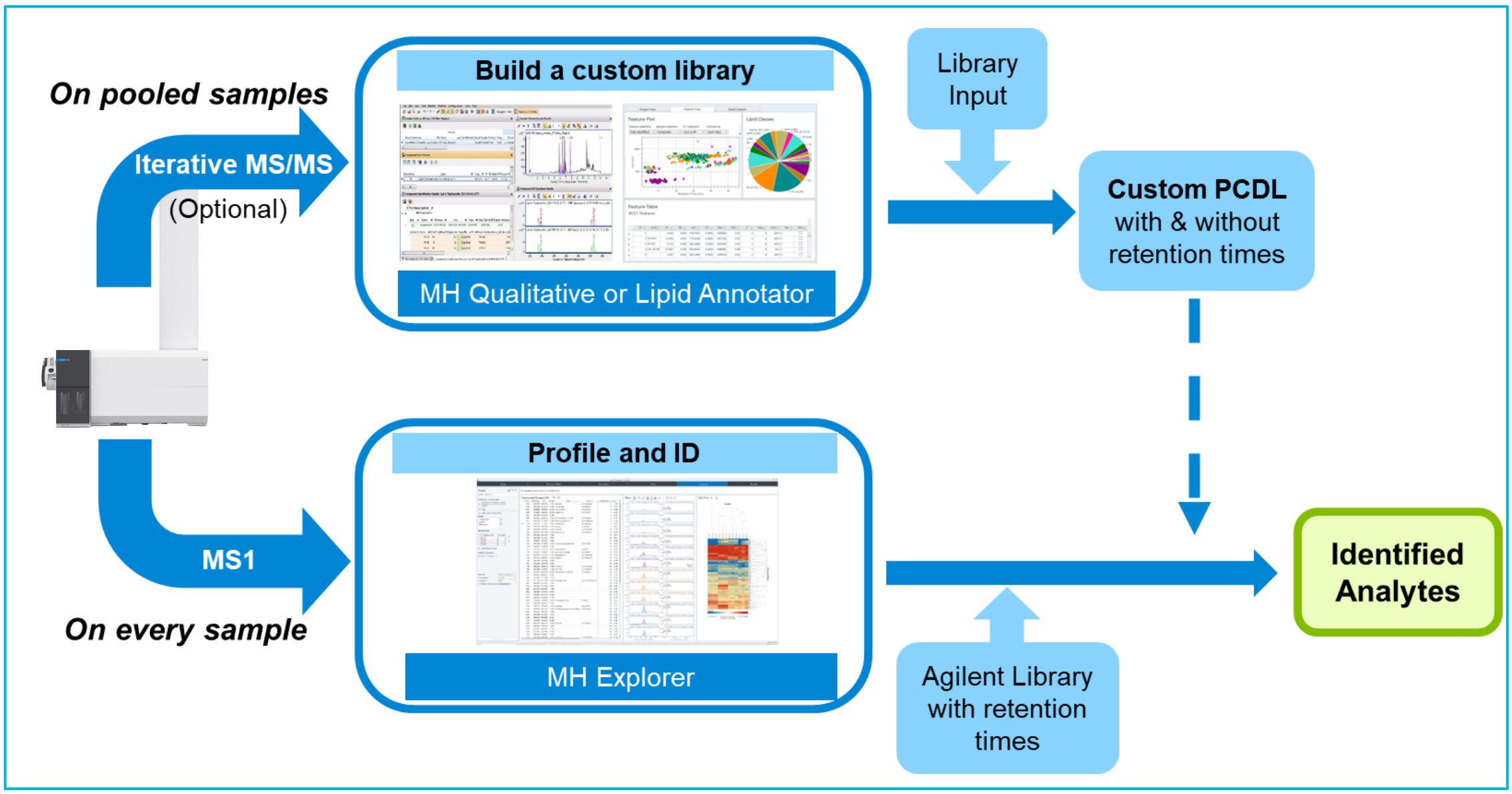

Figure 3. Acquisition and analysis workflow for untargeted metabolomics and lipidomics. MS1 data for each individual sample is collected and analyzed in MassHunter Explorer software for fast and easy feature extraction and statistics. Identification via MassHunter Explorer can be formula generation or analyte ID using a database with or without RT. For this project custom MS/MS and RT libraries for 700+ lipids and 500+ metabolites were initially leveraged for a Tier 1 ID. Custom databases were built with an optional Iterative MS/MS workflow on pooled samples. The lipid data is analyzed in Lipid Annotator which utilizes LipidBlast as its library input and then curates the detected lipids with attention to data quality and annotation level (does not over annotate). The metabolite workflow uses MassHunter Qual 12.0 to extract the Auto MS/MS spectra and identify with a Library input from ChemVista, namely METLIN. Then RTs are easily updated with a new feature in Qual 12 that exports ID'd RTs back to the spectral library for fast RT update capability. In MH Explorer you can select several libraries to allow a tiered ID strategy for different levels of confidence.

Revident LC/Q-TOF Provides Reliable HRMS Data for Untargeted Studies

Pooled QCs that were injected throughout the worklist showed mass and area count stability. Specifically, the mass accuracy for various analytes was <1.27 ppm for metabolites. In a separate experiment this level of mass accuracy held for over seven days of run time with calibrating the MS only at the beginning of the experiment on day one.[4] This suggests a robust instrument that produces reliable results and does not need maintenance or stoppages during data collection.

MassHunter Qual 12.0, Lipid Annotator, and ChemVista Make Custom Library Building Straightforward

Using MassHunter Acquisition 12.0 Intelligence workflows for the iterative experiment also increased the usability of collecting this impactful data. With this new software version method blanks can be automatically analyzed, and blank background features are added to the exclusion list leading to iterative data that is richer in biological identifications. Analysis of this data was made easy with Lipid Annotator which simply extracts and IDs the features using a LipidBlast theoretical library. A custom lipid library is exported from this software. For the metabolites MassHunter Qual 12.0 is used for an Auto MS/MS extraction and Library ID using METLIN or expanded libraries from ChemVista. Here RTs are exported easily back into the library giving a full analyte library with and without RT. Results from the mouse study are summarized in Table 1.

Results and Discussion

MassHunter Explorer is Incredibly Fast and Easy to Use for Feature Extraction, Data Normalization, Statistics and Analyte Identification

Table 1. Summary of Iterative and MS1 Features Extracted and Identified. Existing libraries with spectra and RTs curated with standards are also included since they are part of the analysis workflow.

Experiment Step	Lipid Results		Metabolite Results	
	Pos	Neg	Pos	Neg
Iterative MS/MS Features	3764	2184	5233	4657
Iterative MS/MS ID'd	269	81	2377	1970
Existing Spectral and RT Library	763		523	
Explorer Features Extracted	10470	NA	10795	16204
Explorer Significant Features	612		1094	582
Significant Features ID'd with Libraries	55		414	336

MassHunter Explorer processed all the samples for each experiment in about 1 hour using a standard Revident PC and found over 10,000 features in each data set. This software is easy to use, very fast, and produces publication ready plots for biological interpretation (Fig. 4).

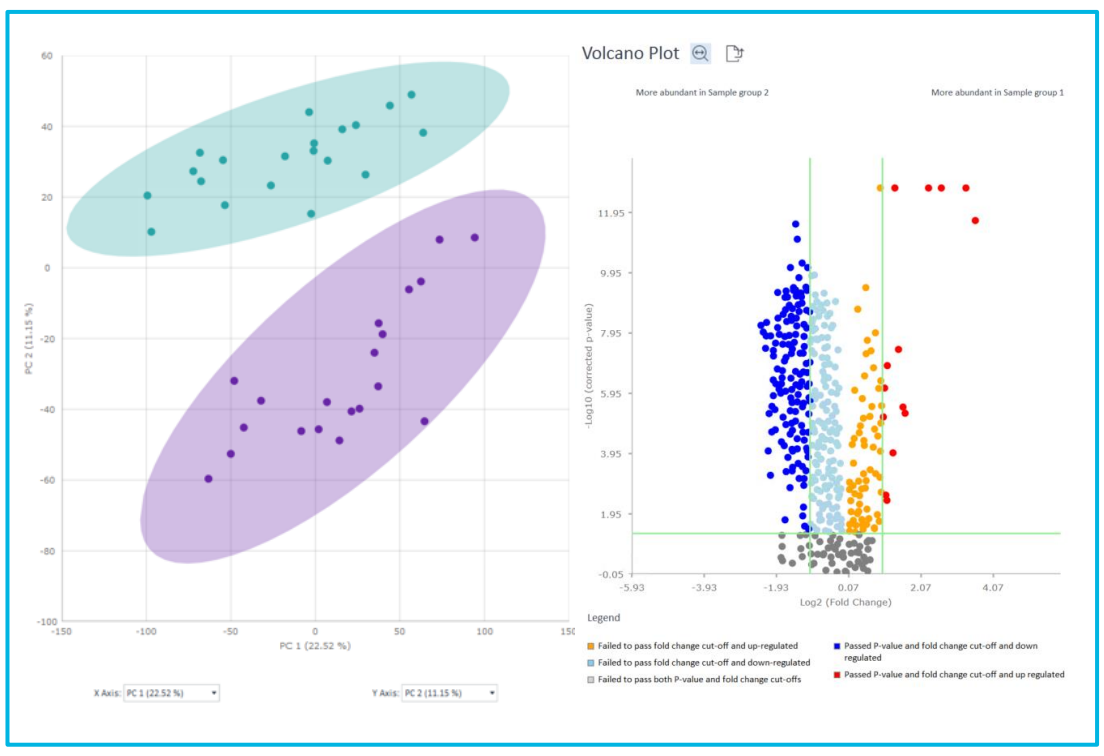

Figure 4. Plots from MassHunter Explorer are publication ready. PCA plot for metabolites shows distinction between the male/female population is inherent (left) and the volcano plot for the lipids show up and down regulated lipids when considering fold change of 2 and p-value of 0.05 (right).

When Unknowns Still Exist, Use SIRIUS CSI:FingerID[5] for MS/MS Structure Identification and Database Searching

When a feature is still unidentified after applying in house databases, its MS/MS spectra can be submitted to SIRIUS CSI:FingerID for structural analysis from the MS/MS fragments and an online database search for similar structures (no online MS/MS spectra needed).

Conclusions

Untargeted Metabolomics Workflows are Much More Routine and Ready to Deploy with Advancements in Hardware, Software, and Supportable Methodologies to Jump Start Your Omics Research

- Combined metabolite and lipid extraction from the same sample is efficient and produces clean extracts.
- Revident LC/Q-TOF produced reliable data with consistently low mass error in each worklist.
- All the methods described here are supportable by Agilent for getting new users set up with the whole workflow or any portion that is needed.
- Identification workflow is comprehensive relying on:
  - Existing MS/MS and RT libraries containing 763 lipid and 523 metabolites for plasma
  - Custom MS/MS library building with improved iterative MS/MS acquisition and software for RT updating
  - SIRIUS CSI:FingerID for unknown MS/MS spectral identification and online searching
- MH Explorer is a very fast software that is easy to use and apply various libraries for tiered identification.

References

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