Application Note

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An End-to-End Untargeted LC/MS Workflow for Metabolomics and Lipidomics

Authors

Sierra D. Durham,
Karen E. Yannell,
Cate Simmermaker,
Genevieve Van de Bittner,
Lee Bertram,
Daniel Cuthbertson, and
Chris Klein
Agilent Technologies, Inc.

Abstract

Untargeted metabolomics and lipidomics LC/MS analyses are key discovery techniques for researchers working to gain insight into biological systems. This application note presents a comprehensive end-to-end workflow covering sample preparation, instrumental analysis, and untargeted data analysis solutions for both metabolomics and lipidomics from the same plasma or mammalian cell sample. Automated sample preparation was conducted using the Agilent Bravo Metabolomics Sample Preparation Platform paired with an Agilent Captiva EMR–Lipid plate, which separates polar metabolite and lipid fractions. Chromatographic separation was achieved using the Agilent 1290 Infinity II bio LC system coupled with an Agilent InfinityLab Poroshell 120 HILIC-Z column for metabolomics, and an Agilent ZORBAX RRHD Eclipse Plus C18 column for lipidomics. Equivalent performance is expected with Agilent 1290 Infinity III bio LCs, and all methods described here are fully compatible with both Infinity II and Infinity III bio LC systems. Metabolite and lipid detection was conducted using the Agilent Revident quadrupole time-of-flight LC/MS system (LC/Q-TOF), which provides excellent resolution, isotope fidelity, and mass accuracy. Untargeted data analysis was performed using Agilent MassHunter Explorer, while custom library building and curation were managed using Agilent MassHunter Qualitative Analysis or Agilent MassHunter Lipid Annotator paired with Agilent ChemVista library manager. Further analysis for identifying any remaining unknowns was facilitated by SIRIUS with CSI:FingerID. The results demonstrate that this untargeted workflow can be used for the reliable extraction, fractionation, separation, detection, analysis, and identification of metabolites and lipids across various compound classes. This easy-to-implement workflow will reduce the time required to adopt omics methodologies in any laboratory.

Introduction

Investigators in life and applied sciences are accelerating adoption of metabolomics and lipidomics techniques to deliver broad insights into the function of biological systems. Metabolomics and lipidomics studies provide details of cellular metabolism, the effects on biological pathways from treatments, or the impacts of environmental toxins. To maximize the impact of these studies, investigators need easy-to-implement and robust workflows. One of the most critical tasks of any metabolomics or lipidomics experiment is the confident identification of analytes. This application note presents a comprehensive end-to-end workflow using the Agilent Revident quadrupole time-of-flight LC/MS system (LC/Q-TOF) for metabolomics and lipidomics, incorporating sample preparation, analyte separation, detection, statistical data analysis, and unknown spectral identification.

Metabolomics and lipidomics experiments are typically categorized as targeted, semitargeted, or untargeted discovery approaches. Agilent has previously published a targeted workflow solution for over 500 metabolites and more than 650 lipids using the Agilent 6495 triple quadrupole LC/MS (LC/TQ).[1,2] Here, we present a complimentary untargeted Q-TOF LC/MS workflow, allowing researchers to expand their analysis scope to a full discovery approach. The Revident LC/Q-TOF provides high resolution at fast acquisition speeds, enabling robust integration, extended dynamic range, and isotopic fidelity for confident identifications.

The untargeted workflow uses automatable sample preparation[3-6] and the same robust HILIC separation[7] as the LC/TQ workflow. The process is supported with highly curated libraries containing accurate masses, MS/MS spectra, and retention times for the corresponding chromatographic methods of over 500 metabolites and more than 650 lipids. Furthermore, the lipid library contains a significant amount of deep structural annotation, identified through an orthogonal experiment. Together with reproducible separation, this feature enables researchers to have more complete lipid information.[8,9]

All these tools combined allow users to make confident metabolite and lipid identifications and generate biological insights quickly. Untargeted data are processed with Agilent MassHunter Explorer for feature finding, identification, and statistical analysis, while SIRIUS with CSI:FingerID is used for further compound identification.[10] This comprehensive LC/Q-TOF workflow provides a jump-start to untargeted analysis of polar metabolites and lipids for biological researchers, with each component adding value to any metabolomics or lipidomics experiment.

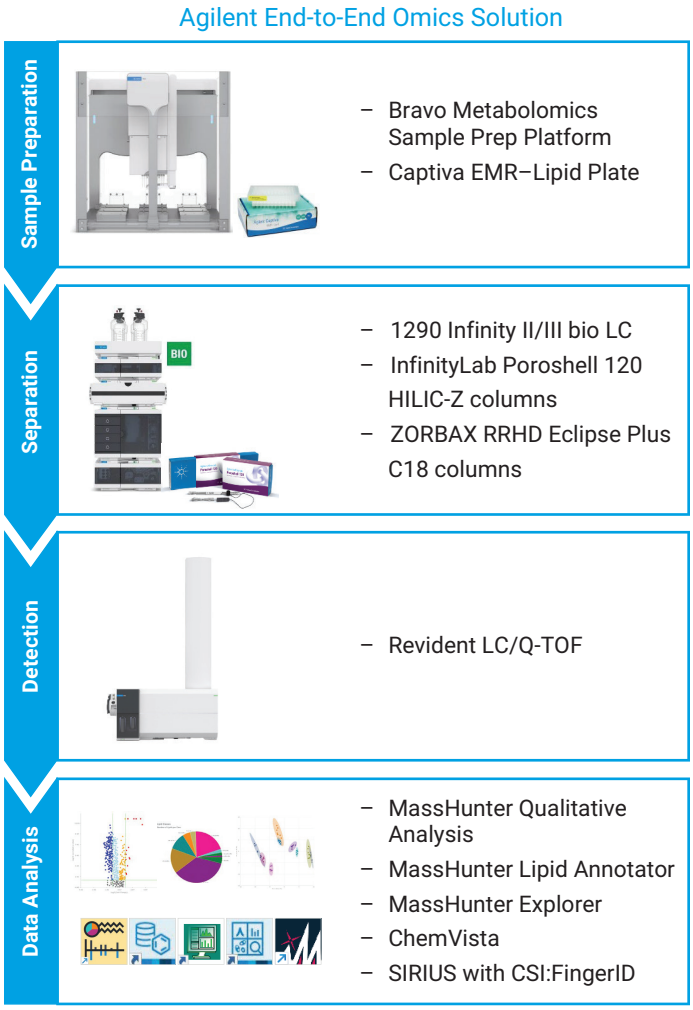

Figure 1. End-to-End untargeted analysis workflow focuses on automated sample preparation of cells or plasma, robust and reproducible HILIC chromatography for separation of polar metabolites or C18 chromatography for lipids using Agilent 1290 Infinity II or Infinity III bio LC systems, Agilent Revident quadrupole time-of-flight LC/MS system (LC/Q-TOF) for confident untargeted analysis, Agilent MassHunter software for statistical and differential analysis, and SIRIUS with CSI:FingerID for structural identification and online database searching of unknowns from MS/MS spectra.

Experimental

Sample preparation

Samples were prepared using the Agilent Bravo Metabolomics Sample Prep Platform and Agilent Captiva EMR–Lipid plates. These tools can be combined to fractionate metabolite, lipid, and protein fractions from the same plasma sample, conserving the sample and better enabling direct biological comparisons from the same aliquot.[3–6,11] For this experiment, metabolites and lipids were extracted from mouse plasma (biological replicates, n = 20 male and n = 20 female, 20 µL per well), as shown in Figure 2. The automated and easy-to-use sample preparation with the Bravo Metabolomics Sample Prep Platform offers high recoveries and reduces sample preparation time. Importantly, automation in this step reduces variability by up to 50%. The resulting lower coefficients of variation improve the statistical power of downstream analyses.[11] Metabolites and lipids can also be cofractionated from cell samples using a similar workflow, as previously described.[4]

Both the metabolite and lipid fractions were dried down for storage. The metabolite fraction was reconstituted with 70% acetonitrile, 20% water, and 10% methanol (100 µL/sample) and spiked with stable isotopically labeled analytes as internal standards for a quality control assessment. The lipid fraction was reconstituted with 90% methanol, 10% chloroform (100 µL/sample), with a combination of sonication and orbital shaking used to aid in redissolution.

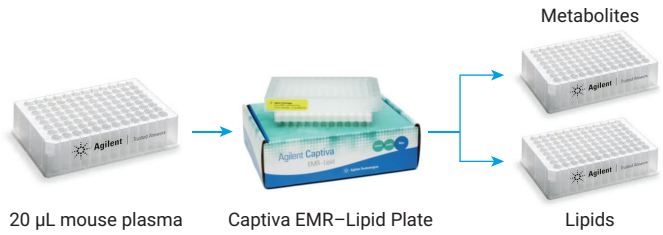

Figure 2. Agilent Captiva EMR–Lipid plates are used to filter out the protein precipitate, capture lipids on the sorbent, and elute the polar metabolites into a collection plate. A second set of solvents releases the lipids into a separate collection plate, allowing for the extraction of both polar metabolites and lipids from the same plasma or cell aliquot. The Agilent Metabolomics Bravo Sample Preparation Platform can perform this solid phase extraction in 96-well plate format using integrated bench top accessories.[3,4]

Separation

Established chromatography for both polar metabolites and lipids on an Agilent 1290 Infinity II bio LC with a standardized configuration have been described in detail previously.[1,7] Briefly, polar metabolites were separated using an Agilent InfinityLab Poroshell 120 HILIC-Z column (2.1 × 150 mm, 2.7 µm) using a method with a total run time of 24 minutes. Lipid separation was carried out on an Agilent ZORBAX RRHD Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm) with a 16-minute method. Both chromatographic methods are fully compatible with Agilent 1290 Infinity III bio LCs, with equivalent results expected on both systems.

The integration of automated sample preparation and robust, standardized chromatography offers a reliable, fully tested methodology for detecting metabolites and lipids in complex matrices. This approach allows predictable and recorded retention times, adding to identification confidence.

Detection

Untargeted metabolite and lipid analysis was performed using the Revident LC/Q-TOF. Untargeted analysis is one of the most challenging mass spectrometry applications, requiring sophisticated instrumentation for excellent analysis. The Revident LC/Q-TOF was specifically developed for this purpose, simultaneously delivering a wide dynamic range, high resolution, isotope fidelity, mass accuracy, and high experimental precision. When all aspects are present, the statistical analysis can yield unambiguous identification of the statistically relevant features.

Iterative data dependent acquisition: Traditional data-dependent (Auto) MS/MS data acquisition is limited by the number of precursors that can be selected for fragmentation during chromatographic elution, which leads to an abundance bias and often misses important low-abundance analytes. Iterative MS/MS analysis overcomes this limitation through an automated workflow that excludes precursors on a rolling basis, allowing sequential injections to provide fragmentation of lower abundance analytes. Iterative analysis on the Revident Q-TOF LC/MS system is facilitated by a built-in automated intelligent reflex workflow. This workflow also allows blanks to be analyzed and added to the iterative exclusion list, resulting in data that are richer in biological identifications.

Iterative metabolomics and lipidomics analyses were performed on pooled sample extracts using the aforementioned chromatography, with Q-TOF source and acquisition parameters as described in previous Agilent publications and summarized in Tables 1 and 2.[12,13] Separate iterative analyses were carried out in positive and negative polarities, with eight iterations collected for each polarity for both lipids and metabolites. This iterative data was used to build custom metabolite and lipid databases with retention times tailored for this sample set, to be applied during MS1 data analysis.

MS1 analysis can be performed without the corresponding iterative analysis. However, adding iterative analysis and custom database building is recommended as best practice, as it facilitates richer compound identifications in the MS1 data.

MS1: Metabolomics and lipidomics analyses were conducted on the corresponding extract for each mouse plasma sample, following previously published methods with slight modifications noted in Tables 1 and 2 to ensure optimal performance of the Revident LC/Q-TOF.[1,2] MS1 metabolomics analysis was performed with two injections per sample, one for each polarity. While only positive polarity MS1 analysis was used for broad coverage of the lipid classes in this study, additional negative polarity MS1 analysis provides complementary coverage and is recommended for maximal lipid identifications.

Table 1. Agilent Jet Stream (AJS) source conditions optimized for the corresponding HILIC metabolomics analysis of fragile analytes and C18 lipid analysis.

Agilent Jet Stream (AJS) Source Parameters		
	HILIC Metabolomics	**C18 Lipidomics**
Ion Mode	AJS, positive/negative	AJS, positive
Gas Temperature	225 °C	320 °C
Drying Gas Flow	9 L/min	8 L/min
Nebulizer Gas	30 psi	45 psi
Sheath Gas Temperature	375 °C	350 °C
Sheath Gas Flow	12 L/min	11 L/min
Capillary Voltage	3,000 V	3,500 V
Nozzle Voltage	500 V	1,000 V

Table 2. Agilent Revident quadrupole time-of-flight LC/MS system (LC/Q-TOF) acquisition parameters for the corresponding HILIC metabolomics analysis of fragile analytes and C18 lipid analysis.

Revident LC/Q-TOF MS Conditions		
	HILIC Metabolomics	**C18 Lipidomics**
Tune	*m/z* 1,700, fragile ion	*m/z* 1,700, stable ion
Acquisition Range	MS1: *m/z* 60 to 1,000 MS2: *m/z* 20 to 1,000	MS1: *m/z* 50 to 1,700 MS2: *m/z* 20 to 1,700
Acquisition Rate	MS1: 4 Hz MS2: 6 Hz	MS1: 3 Hz MS2: 3 Hz
Fragmentor Voltage	100 V	175 V
Skimmer Voltage	45 V	45 V
Octopole 1 RF Vpp	750 V	400 V
Reference Ions	Purine and HP-921	Purine and HP-921

Data analysis

Data-independent acquisition in discovery metabolomics and lipidomics workflows offers distinct advantages. However, the technique results in complex data files. Software solutions are available to simplify these complex files and deliver confident analyte identifications, as shown in Figure 3.

Agilent ChemVista for mass spectra library management: Agilent ChemVista library management software was used to build Personal Compound Database Libraries (PCDL) for targeted analysis. ChemVista supports extensive compound libraries with MS/MS spectra for identification. The software leverages a compound-centric data model based on chemoinformatic underpinnings that ensures compounds are included without duplication, allowing for streamlined merging of multiple libraries. For metabolomics analysis, the curated Agilent HILIC Metabolomics PCDL with over 500 metabolites with retention times, along with the METLIN metabolite and chemical entity library, were used. For lipid analysis, the curated Agilent Lipidomics PCDL with over 650 lipids with retention times was used. Additionally, ChemVista allows users to curate compound lists by importing available third-party spectra from sources such as the Mass Bank of North America (MoNA)[15], Mass Bank of Europe[16], and EPA libraries.

In this application note, a database is used for identifications based on MS1 and retention times, while a library contains MS/MS data that is applied for spectral matching.

Sometimes, a PCDL containing MS/MS spectra may serve as both a library and a database for different identification workflows, depending on the type of information referenced during the identification process.

MassHunter Qualitative Analysis and MassHunter Lipid Annotator for custom library building: For metabolite analysis of iterative data, MassHunter Qualitative Analysis software was used for Auto MS/MS data extraction and library identification with the METLIN metabolite and chemical entity library. Other expanded libraries from ChemVista could be included in addition to or in place of the METLIN library, based on the analytes of interest. MassHunter Qualitative Analysis 12.0 also facilitates the export of retention times from identified features back to the spectral library for easy updating.

For lipid analysis of iterative data, the process was streamlined using MassHunter Lipid Annotator software, which extracts and identifies features using an in silico library, a modified version of LipidBlast, initially developed by Kind *et al.* and further improved by Tsugawa *et al.*[14] After extraction and identification, Lipid Annotator then facilitates the export of a corresponding custom lipid database. Notably, Lipid Annotator curates lipid identification with consideration to data quality and annotation to avoid over-annotation.

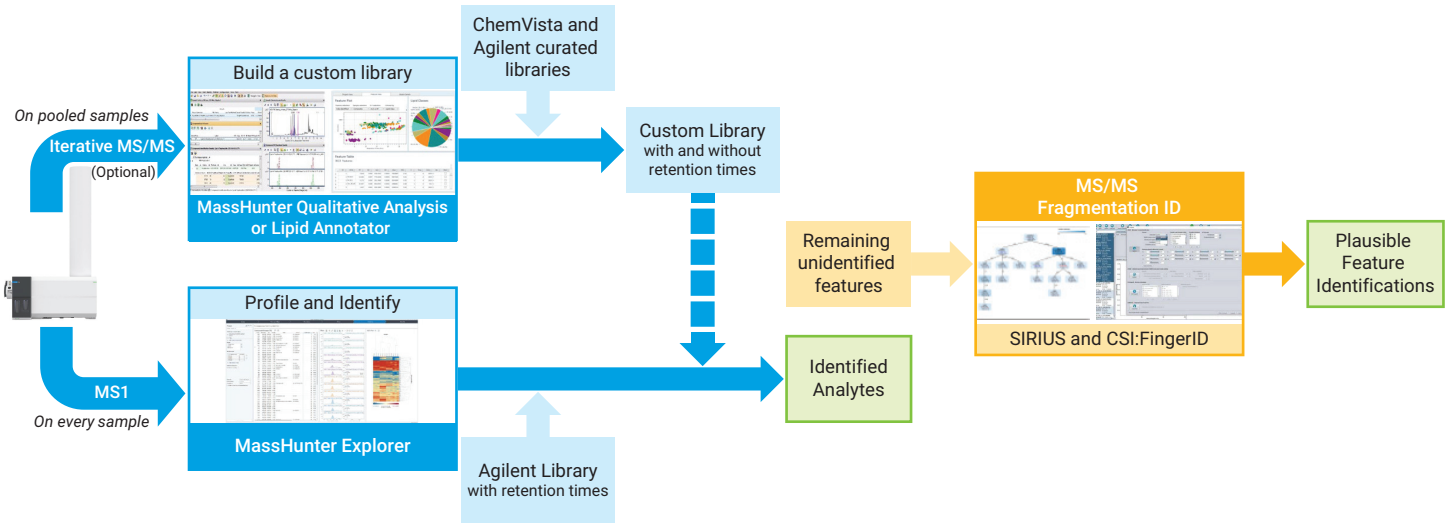

Figure 3. Acquisition and analysis workflow for untargeted metabolomics and lipidomics. MS1 data for each individual sample is collected and analyzed in Agilent MassHunter Explorer software for fast, easy feature extraction and statistics. An additional iterative MS/MS workflow on pooled samples can be performed to build custom databases specific to the experimental sample set. The iterative lipid data are analyzed in Agilent MassHunter Lipid Annotator. The iterative metabolite data are analyzed in Agilent MassHunter Qualitative Analysis 12.0 with extraction of Auto MS/MS spectra and identification with library input from Agilent ChemVista spectral database. Spectral analysis using SIRIUS with CSI:FingerID can be performed on remaining unidentified features to provide additional plausible feature identifications.

The results from iterative data analysis of pooled samples for both metabolites and lipids were used to build custom databases with retention times, curated for this sample set. These databases were then applied during untargeted MS1 data analysis for the identification of significant features. Including the iterative analysis and custom database curation steps is recommended for maximal compound identifications. However, if the iterative portion of the workflow is not performed, MS1 analysis can still be carried out using pre-existing libraries for compound identification.

MassHunter Explorer for profiling and identification: Feature finding and identification of untargeted MS1 data was performed with MassHunter Explorer, which combines feature extraction, statistical analysis, and identification into a single application with an intuitive step-by-step interface. Feature finding with MassHunter Explorer can be jump-started using post-acquisition feature extraction conversion with MassHunter Acquisition 12.0, which performs the first stage of molecular feature extraction post-acquisition as data are being acquired. In MassHunter Explorer, multiple databases can be implemented for identification to facilitate wider compound coverage.

SIRIUS with CSI:FingerID for unknowns analysis: SIRIUS with CSI:FingerID complements the untargeted analysis workflow by translating the unknown features discovered in feature finding, but not identified in spectral library searches, into possible structures. Spectra acquired from additional MS/MS experiments can be dragged and dropped into the SIRIUS interface, resulting in plausible identifications from a structural prediction algorithm, followed by online database searching.[10]

Results and discussion

Revident LC/Q-TOF for confident discovery

The Revident LC/Q-TOF combines excellent isotopic fidelity, mass accuracy, and dynamic range, essential for confident identification in untargeted analysis of complex matrix samples. High isotopic fidelity allows for confident identification of analytes even in the presence of near-*m/z* interferences (Figure 4). Sustained mass accuracy is key for confident analyte identification across multiday runs, as shown in Figure 5. A wide in-spectrum dynamic range facilitates the detection of important low-abundance analytes, even in the presence of high-abundance coeluting molecules (Figure 6).

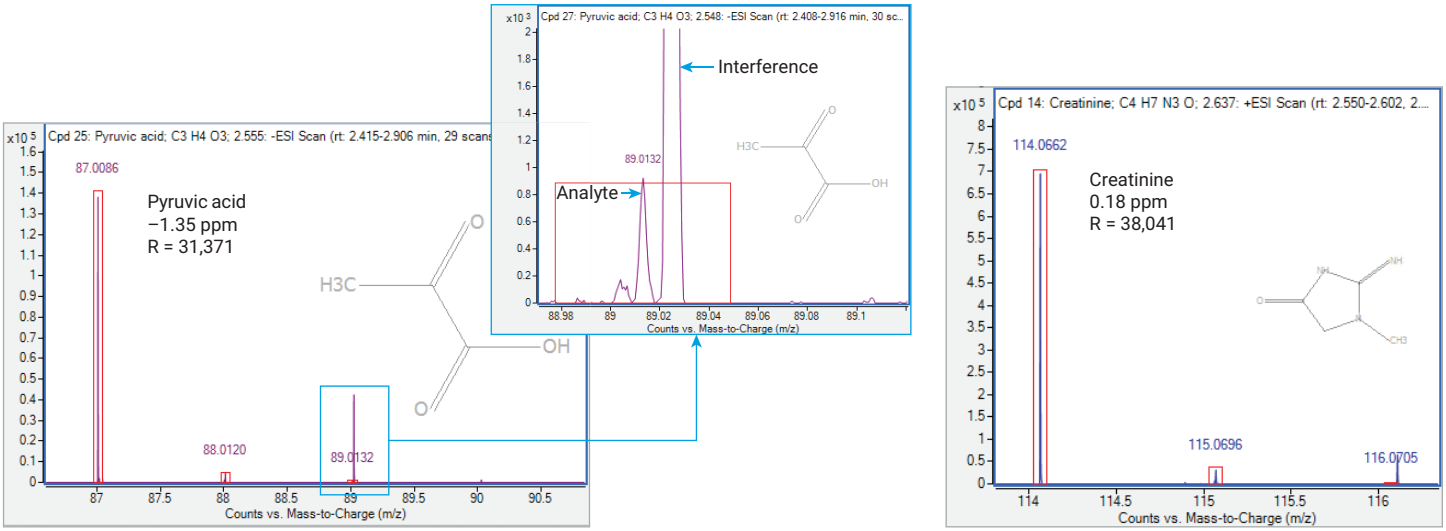

Figure 4. Two examples of confident metabolite identifications from complex matrices using the Agilent Revident quadrupole time-of-flight LC/MS system (LC/Q-TOF). In addition to excellent exact mass measurements, the analyte signal overlaid with the theoretical isotopic pattern (red) shows excellent isotopic fidelity for the analytes. High-resolution mass accuracy is particularly beneficial for pyruvic acid, ensuring the separation of one of its isotopes from a near-mass interference (inset).

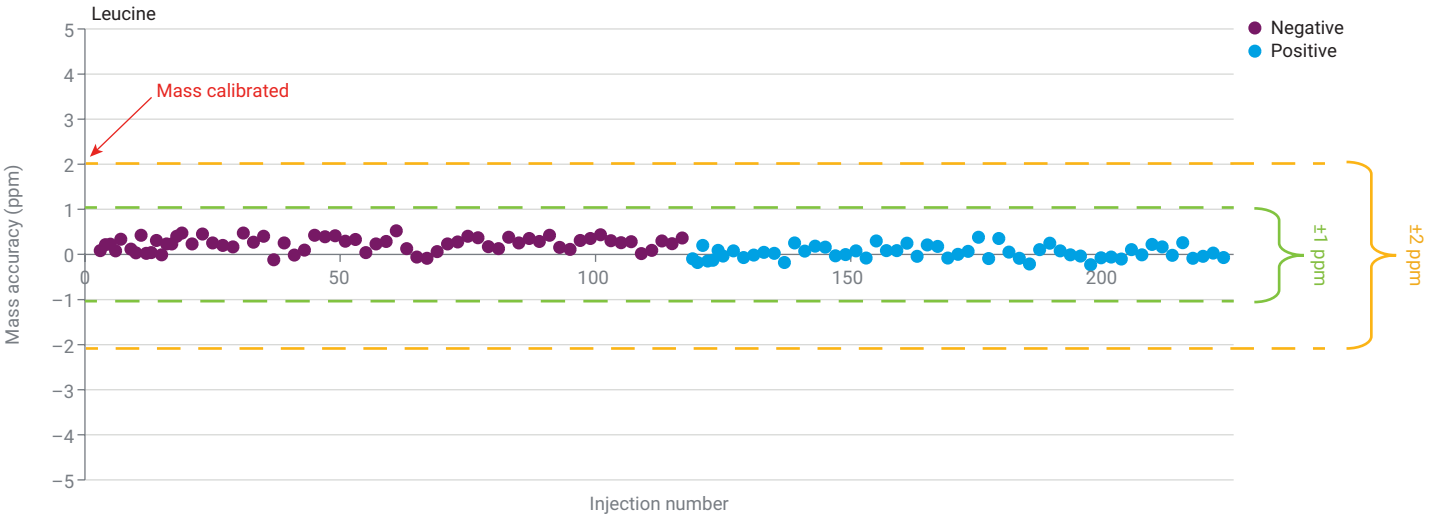

Figure 5. Mass accuracy is a crucial parameter for spectra quality, as it directly influences compound identification. Leucine was used to monitor the mass accuracy (< ±1 ppm) through a series of 225 injections over seven days.

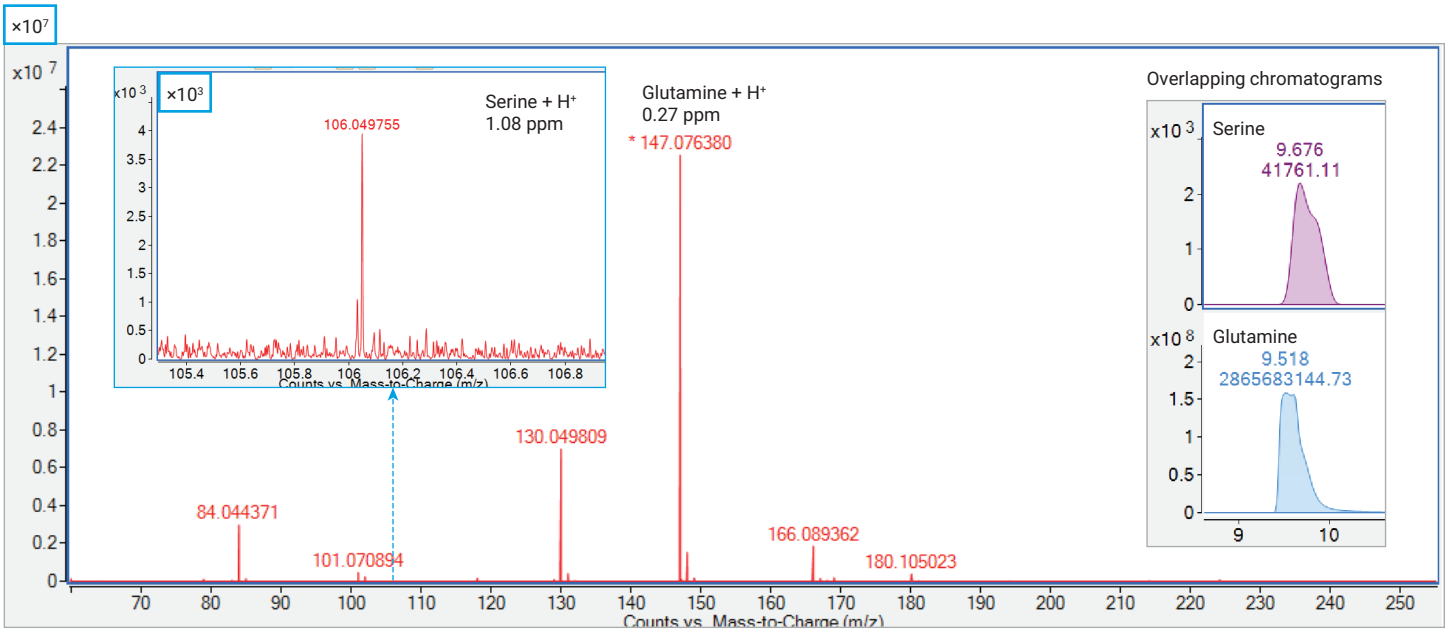

Figure 6. The Agilent Revident quadrupole time-of-flight LC/MS system (LC/Q-TOF) achieves five orders of dynamic range in full spectrum. This figure demonstrates over four orders using glutamine + H^+ and serine + H^+. Notably, despite the high saturation of glutamine + H^+ with over 20 million counts of abundance, mass accuracy remains high. This finding is due to the new Revident detector, which improves peak symmetry across different abundance levels, resulting in consistent mass accuracy. The inset chromatogram shows that both overlapping peaks are well integrated with this detector quality.

Unknowns identification workflow

Library building: Analysis of iterative MS/MS data on pooled metabolite samples using MassHunter Qualitative Analysis with the METLIN library was conducted to create a custom metabolite database for this sample set. Identifications included 693 positive ion and 773 negative ion metabolites.

MassHunter Lipid Annotator was used for a parallel analysis of iterative MS/MS data from pooled lipid samples. Using an in-silico library, a modified version of LipidBlast[14], 269 positive ion and 81 negative ion lipids were identified and added to a custom lipid database for this sample set.

Feature finding, profiling, statistics, and identification: MassHunter Explorer provides fast and easy-to-use feature extraction, data normalization, and statistical analysis, applying existing custom-built databases for analyte identification (Figure 7). The data for all 40 mouse plasma samples in each experiment was processed in approximately 1 hour, resulting in the discovery of over 10,000 features in each metabolite and lipid data set.

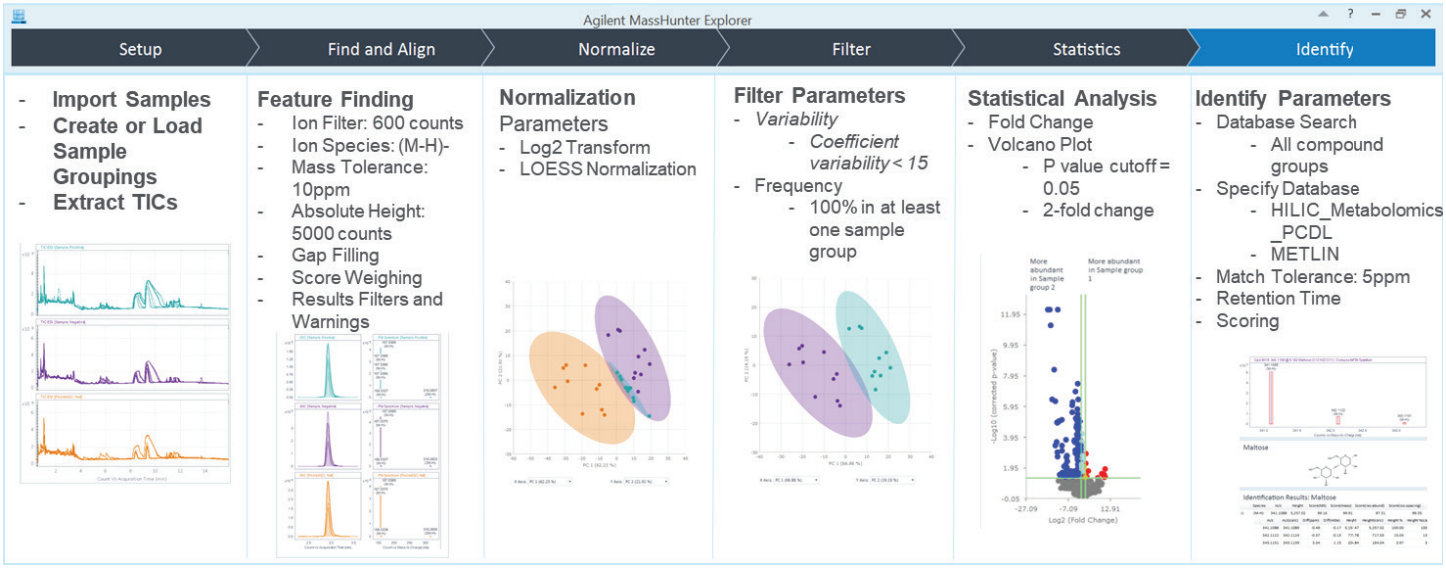

Figure 7. Discovery workflow for differential or biomarker analysis using Agilent MassHunter Explorer. If an unknown still exists, MS/MS data of that analyte can be analyzed in SIRIUS with CSI:FingerID, which produces a molecular fingerprint from the MS/MS data and searches molecular fingerprints computed from online chemical structure databases.

Evaluation of significant features using MassHunter Explorer yielded 1,094 positive ion and 582 negative ion metabolite features that differed significantly (α = 0.05) between the male and female mouse plasma samples (Figure 8A). Of those significant features, 414 positive ion and 336 negative ion metabolites were identified using a combination of the Agilent HILIC metabolomics PCDL with retention times and the custom database built from the corresponding iterative data analysis with MassHunter Qualitative Analysis.

Similarly, 10,470 compound groups, each containing one or more ions, were detected in the lipid dataset using MassHunter Explorer, with 612 features differing significantly between the two mouse populations (Figure 8B). 55 significant lipid IDs resulted from identification with the Agilent lipidomics library with retention times and the custom database built from the corresponding iterative MS/MS data analysis with MassHunter Lipid Annotator.

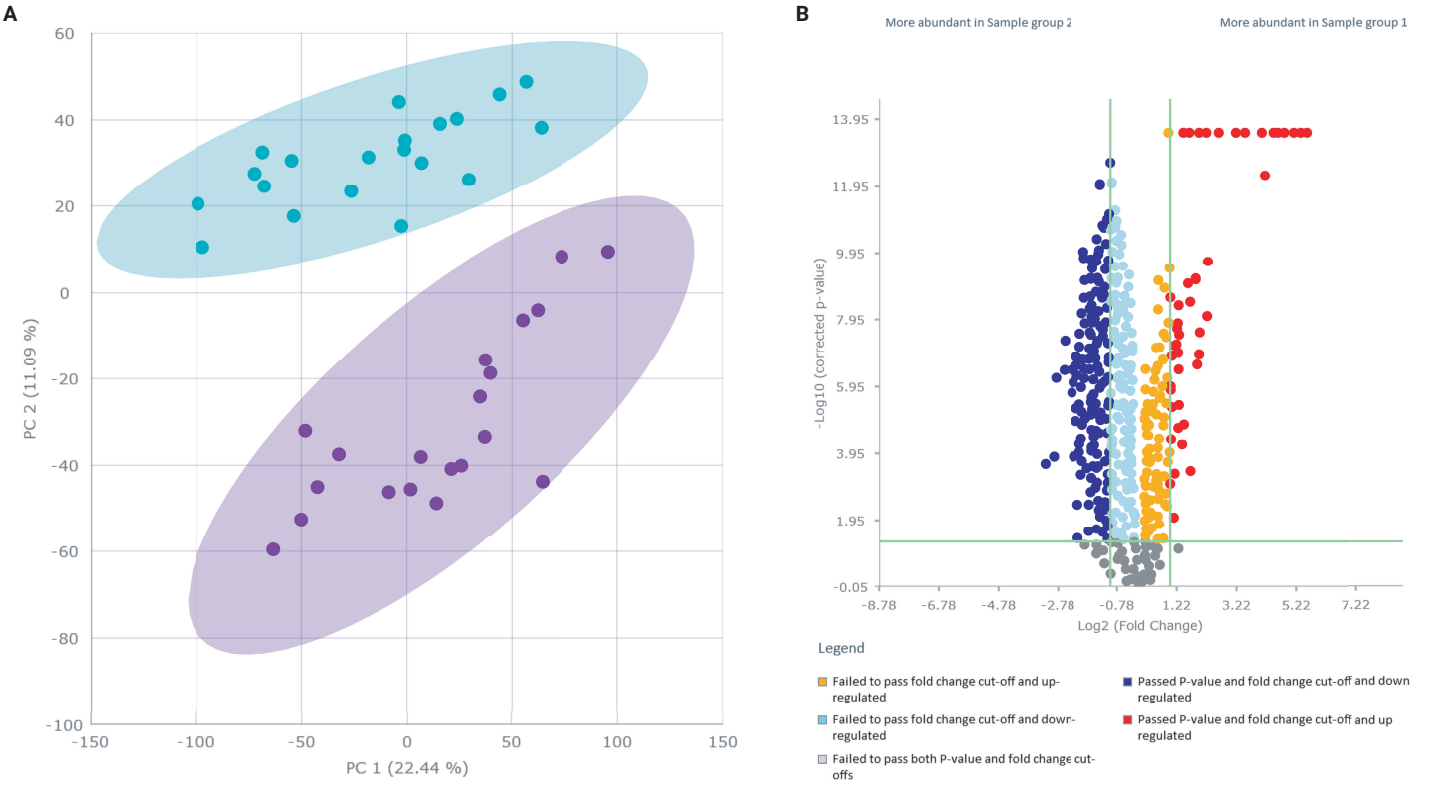

Figure 8. Agilent MassHunter Explorer generates meaningful, publication-ready plots from the data analysis. The PCA plot (A) for metabolites shows an inherent distinction between the male and female populations. The volcano plot (B) for lipids shows up- and down-regulated lipids, considering a fold change of 2 and significance level (p-value) of 0.05.

Unknowns structure identification and database searching: SIRIUS with CSI:FingerID provides easy-to-use molecular formula and structure generation that can be used to expand identification results for significant features that remain unidentified after completing the MassHunter Explorer untargeted data analysis workflow. SIRIUS produces de novo molecular formula matches by comparing experimental spectra with isotope patterns and fragmentation trees.

CSI:FingerID generates structure matches by comparing predicted molecular fingerprints based on the experimental mass spectrum to molecular fingerprints generated from large public structure databases, including PubChem, HMDB, and KEGG. When combined, SIRIUS with CSI:FingerID delivers streamlined compound formula and structure matches that can be used to confirm compound identifications or provide plausible formula and structure identifications for unknowns (Figure 9).

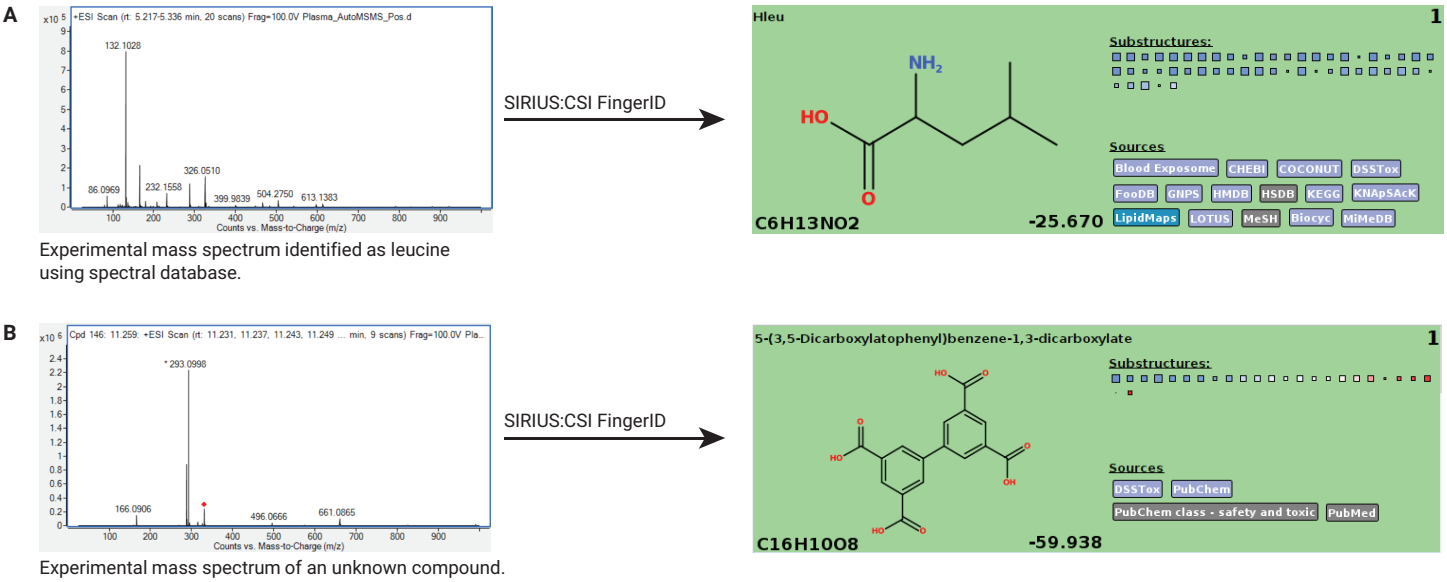

Figure 9. SIRIUS with CSI:FingerID uses experimental MS/MS spectra to generate molecular formula and structure matches which can be used as both a quality control for compounds identified using spectral databases (A) and for identification of unknowns (B).

Conclusion

Agilent solutions offer comprehensive untargeted metabolomics and lipidomics workflows for LC/MS, enabling researchers to more easily achieve biological insights with high confidence. The Agilent Bravo Metabolomics Sample Prep Platform coupled with Agilent Captiva EMR–Lipid plates allowed for efficient extraction of polar metabolites and lipids from the same plasma samples. Robust separation using the Agilent 1290 Infinity II bio LC system paired with the high mass accuracy, isotopic fidelity, and resolution of the Agilent Revident LC/Q-TOF provided reproducible analysis and high confidence in analyte identifications. Similar performance of these methods is also expected with the Agilent 1290 Infinity III bio LC. Database building with MassHunter Qualitative Analysis or Lipid Annotator paired with feature finding, data filtering, statistical analysis, and compound identification in MassHunter Explorer facilitated straight-forward untargeted data analysis, and SIRIUS with CSI:FingerID allowed for additional identification of remaining unknowns. These workflows can be customized to give users the flexibility to answer a wide range of research questions with easy-to-implement methods that accelerate your discoveries.

Learn more about Agilent Revident LC/Q-TOF capabilities and other metabolomics solutions and lipidomics solutions.

References

1. Huynh, K.; *et al.* A Comprehensive, Curated, High-Throughput Method for the Detailed Analysis of the Plasma Lipidome. *Agilent Technologies application note*, publication number 5994-3747EN, **2021**.
2. Yannell, K. E.; *et al.* An End-to-End Targeted Metabolomics Workflow. *Agilent Technologies application note*, publication number 5994-5628EN, **2023**.
3. Van de Bittner, G. C.; *et al.* Automated Fractionation of Low-Volume Plasma Samples for LC/MS Multi-Omics. *Agilent Technologies technical overview*, publication number 5994-7357EN, **2024**.
4. Van de Bittner, G. C.; *et al.* An Automated Dual Metabolite + Lipid Sample Preparation Workflow for Mammalian Cell Samples. *Agilent Technologies technical overview*, publication number 5994-5065EN, **2022**.
5. Sartain, M.; *et al.* Illuminating the Cellular and Molecular Response to Drug Treatment by Combining Bioenergetic Measurements with LC/MS Omics. *Agilent Technologies application note*, publication number 5994-7449EN, **2024**.
6. Agilent Captiva EMR–Lipid Manual Fractionation of Low-Volume Plasma Samples for LC/MS Multi-Omics. *Agilent Technologies method guide*, publication number 5994-7482EN, **2024**.
7. Yannell, K. E.; *et al.* Mastering HILIC-Z Separation for Polar Analytes. *Agilent Technologies application note*, publication number 5994-5949EN, **2023**.
8. Sartain, M.; *et al.* An Interlaboratory Evaluation of a Targeted Lipidomics Method in Plasma. *Agilent Technologies application note*, publication number 5994-6830EN, **2024**.
9. Huynh, K.; *et al.* High-Throughput Plasma Lipidomics: Detailed Mapping of the Associations with Cardiometabolic Risk Factors. *Cell Chem. Biol.* **2019**, *26(1)*, 71–84.e4.
10. Duhrkop, K.; *et al.* Searching Molecular Structure Databases with Tandem Mass Spectra Using CSI:FingerID. *PNAS* **2015**, *112(41)*, 12580–12585.
11. Sartain, M.; *et al.* Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform. *Agilent Technologies application note*, publication number 5994-2156EN, **2020**.
12. Mohsin, S. B.; *et al.* Targeted Data Mining and Annotation of Untargeted High-Resolution Lipidomics Data: A Comprehensive, High-Confidence Workflow. *Agilent Technologies application note*, publication number 5994-7588EN, **2024**.
13. Yannell, K. E.; *et al.* A Comprehensive Untargeted Metabolomics LC/Q-TOF Workflow with and Unknowns Identification Strategy to Identify Plasma Metabolite Shifts in a Mouse Model. *ASMS Poster*, **2022**.
14. Lipidomics Analysis with Lipid Annotator and Mass Profiler Professional. *Agilent Technologies technical overview*, publication number 5994-1111EN, **2020**.
15. MassBank of North America, https://mona.fiehnlab.ucdavis.edu/.
16. MassBank of Europe, https://massbank.eu/MassBank/.

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