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Routine Targeted Metabolomic Panel Analysis from Untargeted Acquisition of Differing Mouse Plasma Populations

Cate Simmermaker, Karen E. Yannell, and Sierra D. Durham

Agilent Technologies, Inc., Santa Clara, CA

Introduction

Data Independent Acquisition for Routine Metabolomic Profiling

Acquisition and analysis of metabolomics data can be made routine and straightforward using data independent acquisition with easy-to-use software for targeted analyte analysis. Untargeted data can be analyzed in a targeted manner evaluating curated compound libraries, specific classes of compounds, matrix specific analytes, or pathways. The inclusion of robust and stable retention times gives additional confidence to metabolite identification. Presented here is an end-to-end workflow solution for routine pathway or library analysis of untargeted plasma metabolomics samples used to investigate differences in mouse populations. Advantages of this workflow for panel assessment includes the ability for retroactive analysis of samples with unlimited scope, unlike the database limitations of targeted workflows.

The LC/Q-TOF is an ideal platform for routine untargeted screening. Acquisition with All Ions methodology includes fragment ions that assist in confident identification of metabolites of interest.

Figure 1. Revident LC/Q-TOF with 1290 Infinity II LC.

Key performance elements of the new Revident LC/Q-TOF are a new detector, giving better mass accuracies over extended concentrations, as well as an increased dynamic range compared to previous instrument generations. In combination with the temperature-inert flight tube, contributing long-time mass stability, the overall mass accuracy has substantially increased. This makes the Revident LC/Q-TOF extremely suitable for routine metabolomic screening as demonstrated in this work.

Experimental

Data Acquisition with Revident LC/Q-TOF

Polar metabolites from plasma (20 µL, mouse) were extracted by Captiva EMR-Lipid plates using an automated liquid handler, 20 male and 20 female. Data was acquired on the iron-free 1290 Bio HPLC and Revident LC/Q-TOF (Table 1 and 2). Samples were spiked with 4 heavy labeled internal standards and pooled QC samples were placed throughout the worklist. Data was acquired using All Ions, an MS Only experiment with three collision energies (0, 10 and 40 V), providing fragmentation information for all analytes to assist in identification without cumbersome targeted analyte lists.[1,2]

Table 1. LC Conditions with 1290 Infinity II Bio LC.

LC Conditions	
Column	Agilent Poroshell 120 HILIC-Z, 2.7 µm, 2.1 x 150 mm (pn 683775-924)
Column temp	15 °C
Injection	4 µL
Autosampler	5 °C
Mobile phase	A = 20mM ammonium acetate, pH 9.3 + 5µM medronic acid in H_2O B = pure ACN
Flow rate	0.400 mL/min
Gradient program	Time %B 0.0 90 1.00 90 8.00 78 12.00 60 15.00 10 18.00 10 19.00 90 23.00 90

Data was analyzed using MassHunter Quantitative Analysis 12.1 and the LC Screener Tool. Compounds were screened using mass accuracy, mass match score, RT, verified fragment ions and coelution of qualifiers with the quantifier.

Table 2. Source parameters with Revident LC/Q-TOF.

Parameters	Value
Ion mode	AJS, positive/negative
Gas temperature	225 °C
Drying gas flow	9 L/min
Nebulizer gas	30 psi
Sheath gas temperature	375 °C
Sheath gas flow	12 L/min
Capillary voltage	3000 V
Nozzle voltage	500 V

Dynamic Range, Resolution, Isotopic Fidelity and Mass Accuracy in Complex Matrix Required for Confident Screening

The Revident LC/Q-TOF offers significant factors that contribute to identification confidence in untargeted screening when analyzing complex matrix samples.

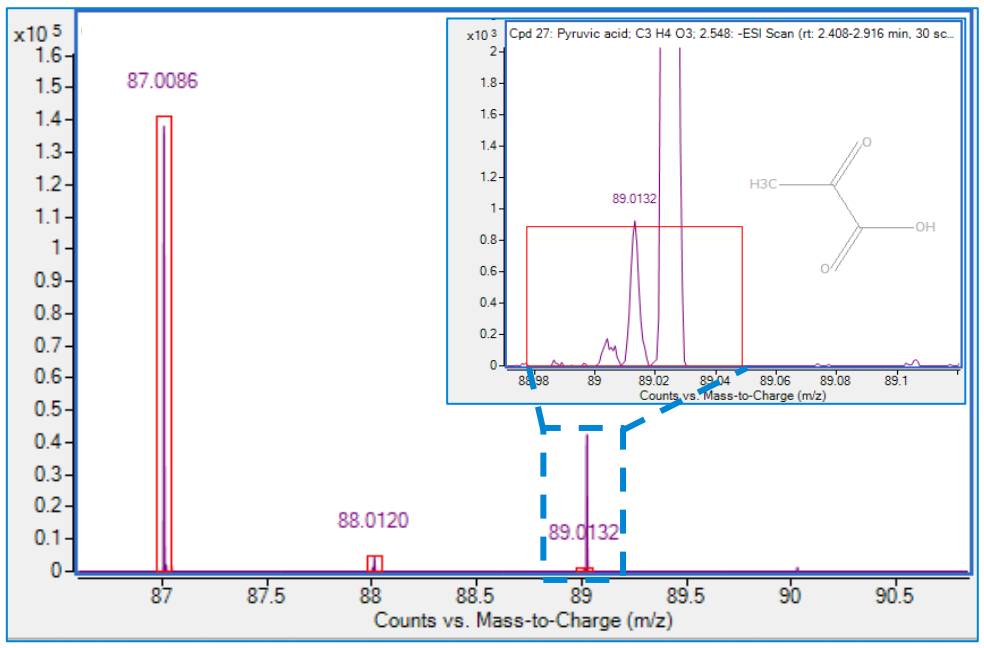

Figure 2. **Isotopic Fidelity** and **Resolution** of pyruvic acid in plasma, R = 31, 371 and mass accuracy = -1.35 ppm.

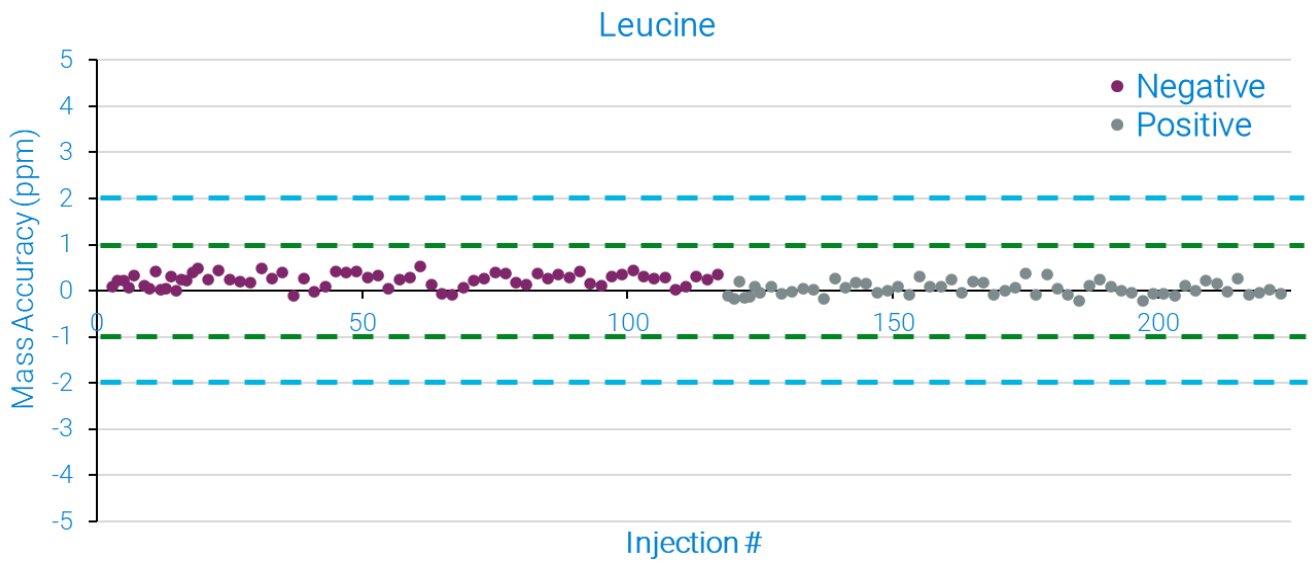

Figure 3. **Mass Accuracy** for leucine in plasma for 225 injections over 7 days without recalibration in both positive and negative modes.

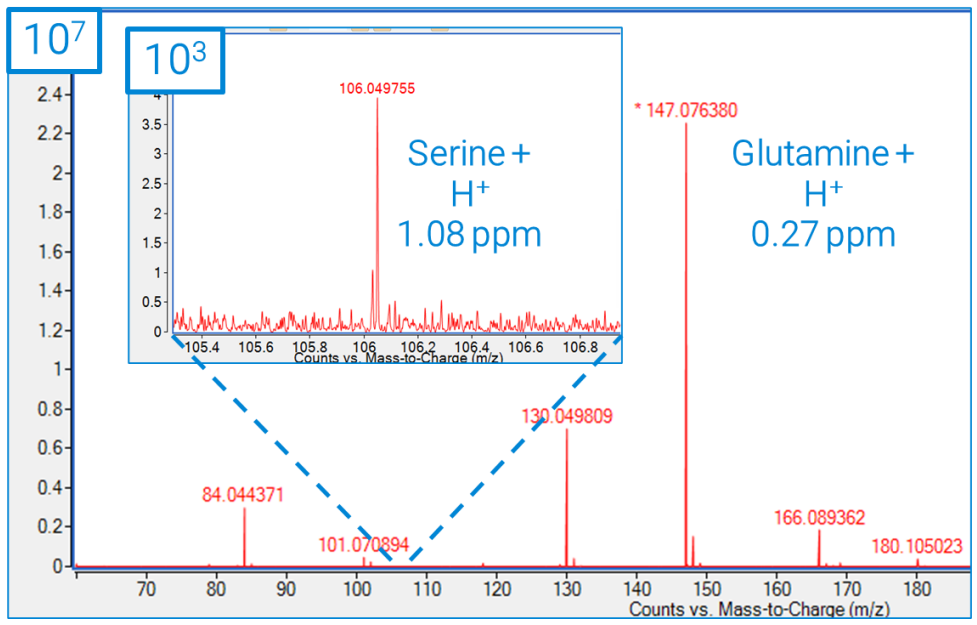

Figure 4. **Dynamic Range** of 4 orders of magnitude between coeluting serine and glutamine.

Routine Metabolomic Profiling with LC Screener Tool in MassHunter Quantitative Analysis Software

Using MassHunter Quantitative Analysis 12.1 with the LC Screener Tool, samples were mined for metabolites using a curated spectral library organized with ChemVista, Agilent's library management software. Libraries for comparison included 377 compounds with 1219 spectra and 446 compounds with 1540 spectra for positive and negative mode, respectively, including 522 metabolites in total. These libraries were curated with RT specific to the HILIC metabolomics workflow.[1,2]

The screening methodology reports the confident presence, questionable presence, or absence of compounds from the library in samples. The report conveniently highlights the compound status with green, orange, or red flags. A compound labeled as present fulfills all the custom qualifier settings, a questionable compound has one outlier that falls outside, and absent compounds fall outside of two or more outlier restrictions. The resulting table for each sample gives a clear summary of the compound results (Figure 6).

Table 3. LC Screener Outlier Parameters.

LC Screener Outlier Parameters	Values
Retention Time Window	10%
Minimum S/N	3
Coelution Score Limit	70
Mass Accuracy Limit	5 ppm
Mass Match Score Minimum	60
Number of Verified Ions Minimum	2

The outliers used for screening include retention time, minimum signal to noise, fragment ion coelution, mass accuracy, mass match score and number of verified ions (Table 3). The LC Screener Tool is an easy way to mine untargeted data for confident identifications.

Identification of metabolites is enhanced with the use of All Ions acquisition. Fragments can be easily evaluated for coelution with the molecular ion using MassHunter Quantitative Analysis 12.1, which combines peak width, peak symmetry, and retention time to generate a score out of 100 (Figure 5 top).

Evaluation of fragment ions is completed at specified collision energies, as indicated by the imported library. All libraries were created utilizing the new ChemVista, which allows for the importing of 3rd party spectra to even further curate and collaborate for efficient library building.

Results and Discussion

Screening Large Metabolite Libraries for Routine Analysis

Leveraging the LC Screener tool (Table 3) embedded into MassHunter Quantitative Analysis 12.1, the subset of samples was quickly evaluated for compound presence or absence. Overall, screening resulted in 128 compounds defined as identified, 101 compounds a possibly present, and 294 compounds as absent from both positive and negative modes. Of the 128 compounds that were present in the samples the data was of high quality and reproducible. 101 compounds were under 20% area RSD with an average area RSD of 14% for pooled QC samples. Over all samples the RT RSD average was 0.81%. 113 of the 128 present compounds had a Mass Match Score over 80 of a possible 100 with an average score of 93, the Mass Match Score combines mass accuracy, isotope abundance, and isotope spacing into a score out of 100. Mass accuracy is of upmost importance in screening, all the tracked average mass accuracies were within +/- 5 ppm with an average mass accuracy of -0.35 ppm from over 10,000 data points.

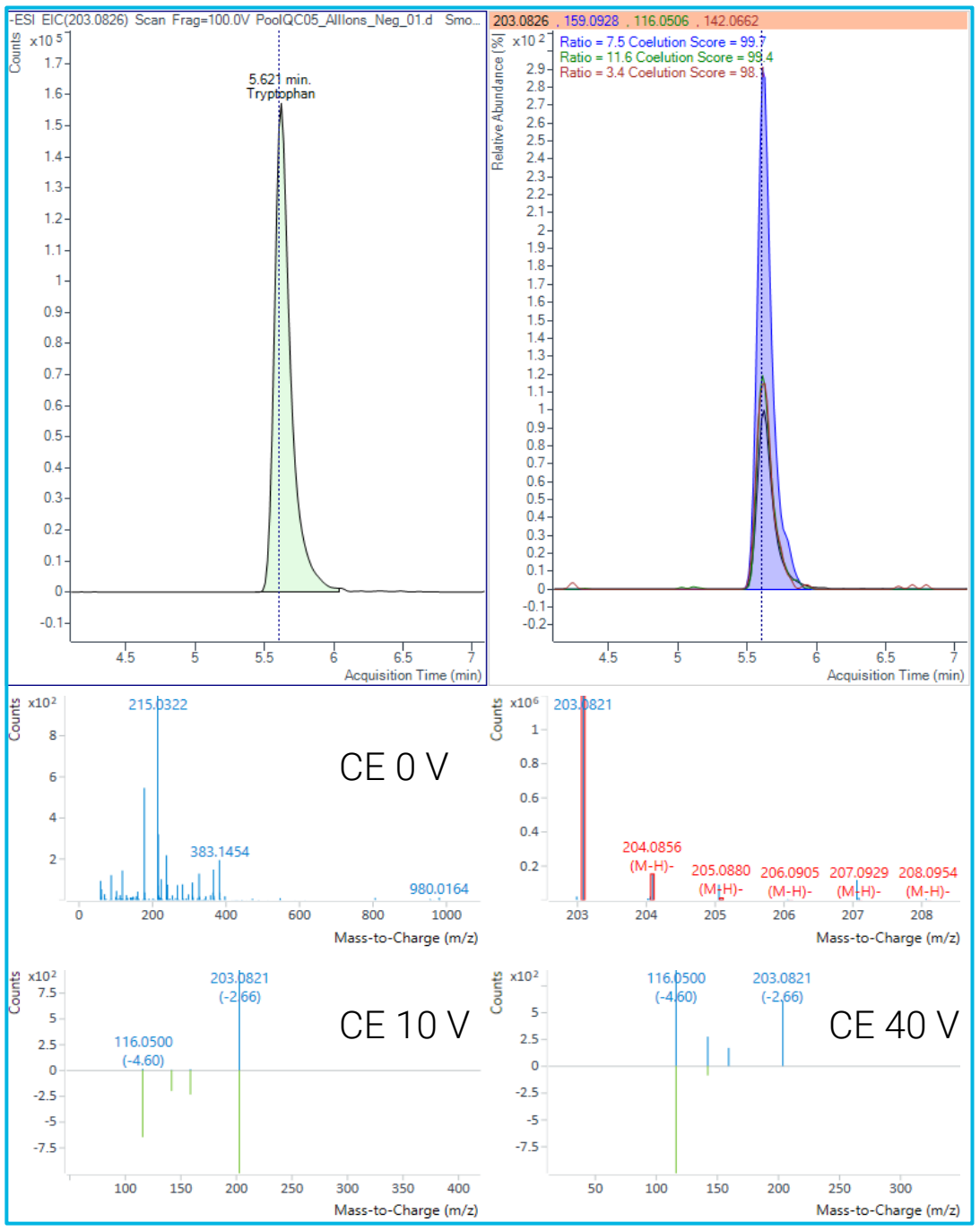

Figure 5. The information provided by the LC Screener tool includes the 4 lower spectra for each compound coordinated to quantifier and qualifier chromatograms.

Application of Metabolite Screening for Differential Analysis

The results of MassHunter Quantitative Analysis can be alternatively analyzed in a statistical analysis workflow. With the same set of data files an analyst can work through an untargeted feature extraction and identification or a targeted screening.

Targets | Suspects | Previous Sample | PoolQC05_AllIons_Neg_01.d | Next Sample | 109 | 102 | 235 | Total: 446

Status	Promoted	Compound Name	CAS#	Formula	R.T.	R.T. Diff.	Mass Match Score	Target Ion	Mass Accuracy	# of Verified Ions	Area	Height
✓	☐	Proline	147-85-3	C5H9NO2	6.887	0.013	99.6	114.0561	-0.6835	3	2460634.8	310344.2
✓	☐	Pyridoxal	66-72-8	C8H9NO3	1.841	0.034	84.0	166.0510	4.1089	3	178442.5	29849.1
✓	☐	Pyridoxamine	85-87-0	C8H12N2O2	6.520	0.025	92.8	167.0826	-0.8948	2	10942.7	2965.7
✓	☐	Pyrocatechol	120-80-9	C6H6O2	0.842	0.007	87.6	109.0295	-1.2773	2	216364.5	44883.4
✓	☐	Ribose	50-69-1	C5H10O5	2.407	0.017	96.2	149.0455	-1.6718	3	763593.5	59624.5
✓	☐	Serine	56-45-1	C3H7NO3	9.434	0.008	99.7	104.0353	-0.9495	2	1287283.8	177034.7
✓	☐	Succinic acid	110-15-6	C4H6O4	11.083	0.003	88.5	117.0193	-0.0684	3	5874657.8	1011497.5
✓	☐	Succinic semialdehyde	692-29-5	C4H6O3	4.256	0.020	99.1	101.0244	-2.9817	2	244451.5	39259.5
✓	☐	Taurine	107-35-7	C2H7NO3S	7.020	0.080	99.0	124.0074	-0.7571	5	6963057.1	627871.2
✓	☐	Taurocholic acid	81-24-3	C26H45NO7S	3.256	0.052	97.5	514.2844	-0.2502	3	4521555.5	207202.3
✓	☐	Threonine	72-19-5	C4H9NO3	8.702	0.049	95.0	118.0510	0.1575	2	3212265.4	590594.1
✓	☐	Thymidine (dT)	50-89-5	C10H14N2O5	1.508	0.000	99.2	241.0830	-1.2398	3	756546.2	87010.0
✓	☐	trans-Aconitic acid	4023-65-8	C6H6O6	11.266	0.034	99.9	173.0092	0.5746	3	1450559.1	248479.5
✓	☐	Trehalose	99-20-7	C12H22O11	9.917	0.227	83.4	341.1089	0.2293	5	29603.7	2803.4
✓	☐	Tryptophan	73-22-3	C11H12N2O2	5.621	0.017	98.4	203.0826	-2.6609	4	1286837.2	157292.6
✓	☐	Tyrosine	60-18-4	C9H11NO3	6.620	0.005	99.7	180.0666	-0.8323	7	2258761.3	297118.1
✓	☐	Uric acid	69-93-2	C5H4N4O3	6.187	0.022	94.9	167.0211	-1.8001	2	72654.5	13469.3
✓	☐	Uridine	58-96-8	C9H12N2O6	2.174	0.016	99.2	243.0623	-1.5558	6	1774299.0	212434.7
✓	☐	Urocanic acid	104-98-3	C6H6N2O2	7.802	0.023	99.8	137.0357	-1.2439	2	106274.7	10808.3
✓	☐	Valine	72-18-4	C5H11NO2	6.670	0.014	99.7	116.0717	-1.1900	2	4641020.2	557705.2

Figure 6. LC Screener Tool results in easy to navigate table with clear color coordinated display.

Statistical analysis with MassHunter Explorer was used to review difference between the two sample populations. Of the 128 presently detected metabolites 12 compounds were identified as differentiating by two-fold with a p-value of <0.05.

For more information on this untargeted analysis workflow please visit ThP085: *Uncovering More Biological Insights in Your Samples with Routine LC/Q-TOF Workflows for Metabolites and Lipids*.

Conclusions

Workflow for the Routine Screening of Metabolites with Confidence from Fragmentation Information

Easy Workflow Setup > Untargeted Metabolite Acquisition > Targeted Mining of Metabolite Pathways

- Clear and concise software workflow for quick screening analysis of metabolites libraries and classes
- 100+ metabolites detected and screened in mouse plasma samples
- Supporting fragmentation information for confident identification with data independent acquisition

References

1 Yannell, KE et al. An End-to-End Targeted Metabolomics Workflow. Agilent Application Note 5994-5628EN. 2023.

2 Yannell, KE et al. A Comprehensive Untargeted Metabolomics LC/Q-TOF Workflow with an Unknowns Identification Strategy to Identify Plasma Metabolite Shifts in a Mouse Model. ASMS, 2022.

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