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A Comprehensive Untargeted Metabolomics LC/Q-TOF Workflow with an Unknowns Identification Strategy to Identify Plasma Metabolite Shifts in a Mouse Model

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Introduction

Combined Plasma Sample Prep to Analyte ID Workflow for Untargeted Metabolomics

Untargeted metabolomics is an approach often employed by researchers interested in identifying biological perturbations. Herein, is a workflow for profiling polar metabolites that includes all untargeted metabolomics workflow steps, from mouse plasma sample preparation to statistical analysis and analyte identification. We build upon a reproducible, high-recovery automated sample preparation method and a robust, stable HILIC chromatography method previously validated as a component of a targeted metabolomics platform. We demonstrate this comprehensive workflow is a robust solution for untargeted discovery metabolomics and unknown identification on a high-resolution mass spectrometer.

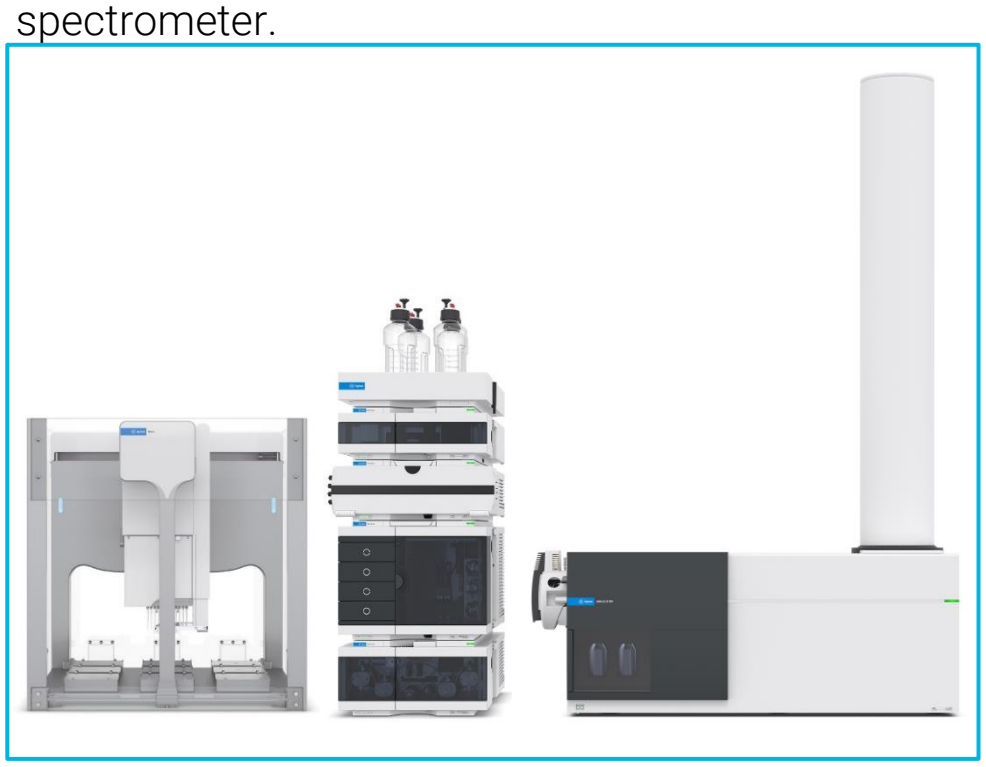


Figure 1: Instruments used for untargeted metabolomics workflow – Metabolomics Bravo platform (left), 1290 Infinity II Bio LC (center), and 6546 LC/Q-TOF which has simultaneous high resolution, isotopic fidelity and extended dynamic range (right).

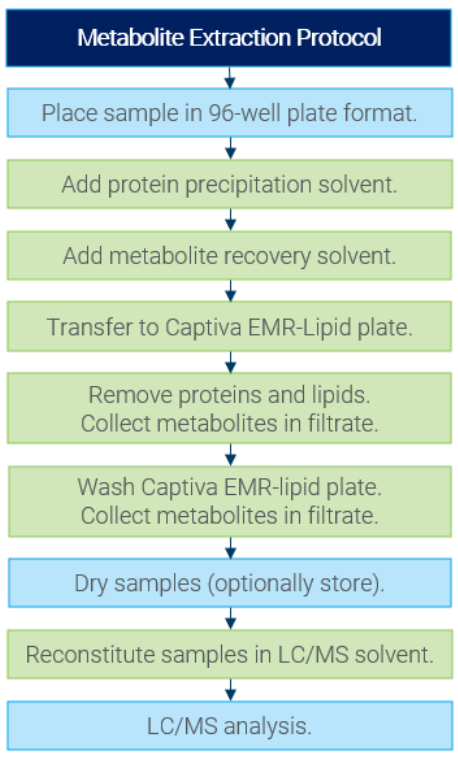


Figure 2: Plasma workflow used to extract metabolites with high recoveries from 20 µL sample while removing lipids and proteins.

Experimental

Bravo Metabolomics Platform Selectively Extracts Metabolites from Plasma Reproducibly and with High Recovery[1]

A previously described solid phase extraction sample preparation method for plasma (Fig 2) was deployed using a Metabolomics Bravo platform. A transferable, robust HILIC chromatography method was utilized with a high-resolution LC/Q-TOF (Fig 3).[2] A custom PCDL for the HILIC chromatography was created from neat standards The workflow was applied to 10 DIO C57BL/6J (obesity model) mouse plasma and 10 controls. All Ions data acquisition with an acquisition rate of 6 Hz and 3 CE was used for acquisition. Analysis is outlined in Fig 5.

LC Conditions		
Column	Agilent Poroshell 120 HILIC-Z, 2.1 x 150 mm 2.7 um, PN 683775-924	
Column temperature	15 ºC	
Injection volume	2 µL	
Autosampler temp	5 ºC	
Needle wash	Standard Wash, 10 sec, IPA:ACN:H2O 1:1:1	
Mobile phase*	A = 20 mM ammonium acetate, pH 9.3 + 5 µM medronic acid water B = ACN	
Flow rate	0.400 mL/min	
Gradient program	Time	%B
	0.00	85
	1.00	85
	8.00	75
	12.00	60
	15.00	10
	18.00	10
	19.10**	85
	23	85
Total Run Time	24 min	

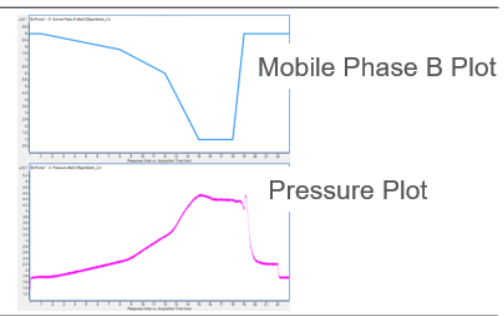


Figure 3: LC conditions produced retentions times with RSD <5% over an 11-day experiment and can be transferred to other systems.[2] *Detailed protocols for column prep and buffer prep ensure that the retention times are reproducible across different labs and skill levels. **0.5 mL/min flow rate used to re-equilibrate the column faster.

AJS Parameters	
Gas temperature	225 ºC
Drying gas flow	9 L/min
Nebulizer gas	30 psi
Sheath gas temperature	375 ºC
Sheath gas flow	12 L/min
Capillary voltage	3000 V (+ and -)
Nozzle voltage	500 V (+ and -)
Q-TOF Parameters	
Tune (Fragile)	m/z 1700
Fragmentor	100 V
Skimmer	45 V
Oct 1 RF Vpp	750 V
Ref Ions	Purine and HP-921

Figure 4: Source conditions for Agilent Jet Stream (AJS) and TOF settings for small polar metabolites.

Analysis Built on Reproducible HILIC-Z Chromatography for Easy Transferability of Methods to Other Labs

(1) All Ions Data Acquisition with 6546 LC/Q-TOF

(2) Profinder Batch Recursive Feature Extraction on sample and pooled QCs (2146 features, neg ion mode)

(3) Statistical analysis in MPP: t-test (p=0.05), fold change (>5, abundance>5000). 47 feature remaining

(4) IDBrowser in MPP for ID with HILIC custom PCDL and Metlin PCDL, 30 of 47 analytes ID'd

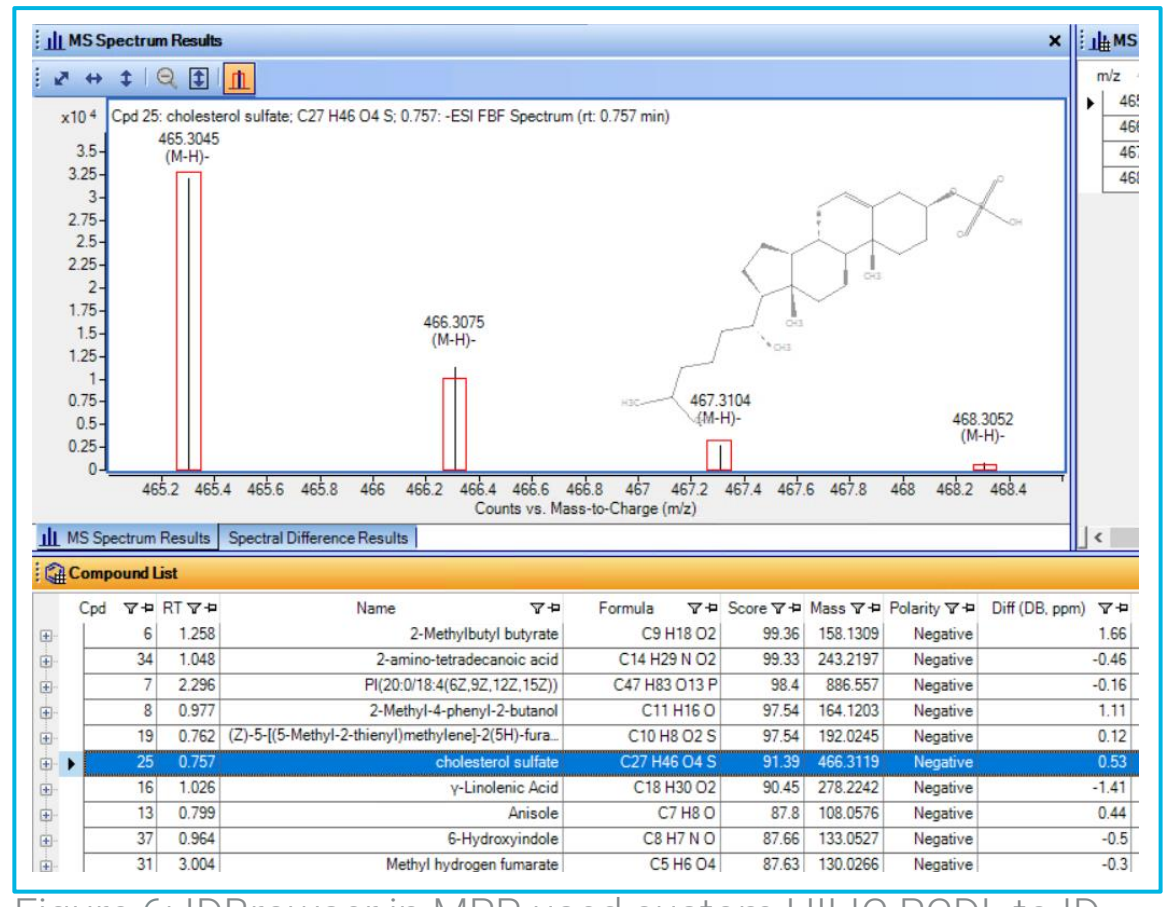
(5) Targeted MS/MS reinjection for unknowns with ID using SIRIUS . 5 additional analytes ID'd (35 of 47 in total)

Figure 5: Workflow for untargeted metabolomics from data acquisition to unknown analyte ID.

The individual samples from both the control and obesity mouse model were randomized and injected with pooled QC samples. Isotopically labeled internal standards were post spiked in each sample and those analytes had a mass accuracy average of <0.5 ppm and RT delta of 0.02 min (n=50). The RT stability and previously shown transferability of the chromatography allowed the data analysis workflow described in Fig 5 to use a custom built PCDL. It contained 542 analytes in it where 506 had spectra and RT. This method and PCDL can be used across labs to aid identification of polar metabolites.

Analytes were extracted with a batch processing feature extraction software MassHunter Profinder, and Mass Profiler Professional (MPP) was the statistical software employed to highlight altering metabolites between the two mouse groups. The built-in IDBrowser was used to easily identify analytes directly within the same software (Fig 6).

6546 LC/Q-TOF Produced Robust Data Set with Low Mass Error and Isotopic Fidelity Aided Confidence in Analyte ID.



Cpd	RT	Name	Formula	Score	Mass	Polarity	Diff (DB, ppm)
6	1.258	2-Methylbutyl butyrate	C9 H18 O2	99.36	158.1309	Negative	1.66
34	1.048	2-amino-tetradecanoic acid	C14 H29 N O2	99.33	243.2197	Negative	-0.46
7	2.296	PI(20:0/18:4(6Z,9Z,12Z,15Z))	C47 H83 O13 P	98.4	886.557	Negative	-0.16
8	0.977	2-Methyl-4-phenyl-2-butanol	C11 H16 O	97.54	164.1203	Negative	1.11
19	0.762	(Z)-5-[(5-Methyl-2-thienyl)methylene]-2(5H)-fura...	C10 H8 O2 S	97.54	192.0245	Negative	0.12
25	0.757	cholesterol sulfate	C27 H46 O4 S	91.39	466.3119	Negative	0.53
16	1.026	γ-Linolenic Acid	C18 H30 O2	90.45	278.2242	Negative	-1.41
13	0.799	Anisole	C7 H8 O	87.8	108.0576	Negative	0.44
37	0.964	6-Hydroxyindole	C8 H7 N O	87.66	133.0527	Negative	-0.5
31	3.004	Methyl hydrogen fumarate	C5 H6 O4	87.63	130.0266	Negative	-0.3

Figure 6: IDBrowser in MPP used custom HILIC PCDL to ID altering metabolites in the sample set.

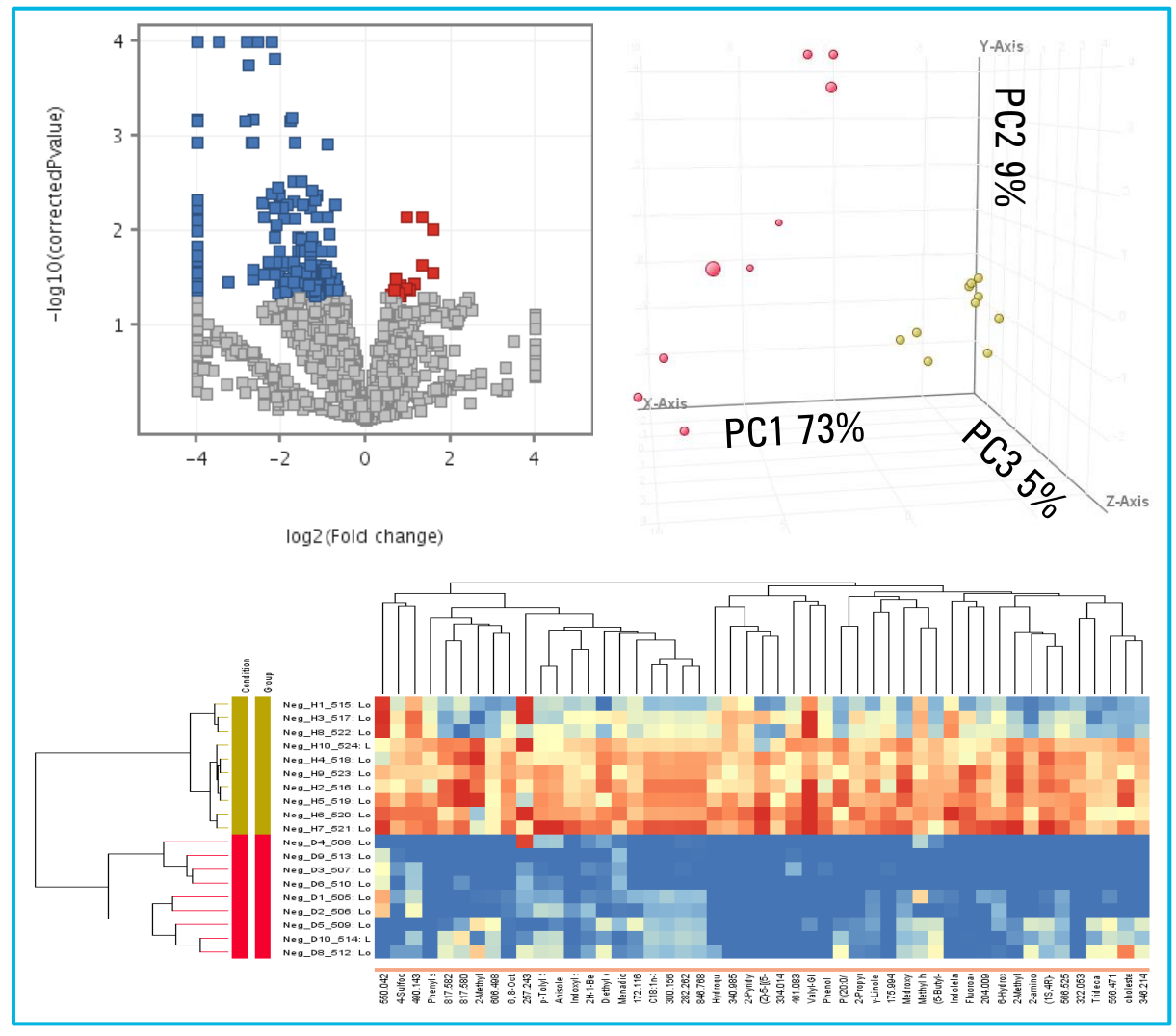


Figure 7: Volcano plot (top left) filtered all the features to just 47 which had a p=0.05 and fold change >5. On those features separation was achieved in the PCA plot (top right), and the hierarchical cluster plot (bottom) showed the dysregulation and relationship of each analyte in the sample.

Results and Discussion

MPP Statistical Software and Embedded IDBrowser Give Biological Insights into Samples with Ease.

Unknown Unknowns are Identified Using MS/MS Data and SIRIUS Software with Links to Databases and Classification Breakdown

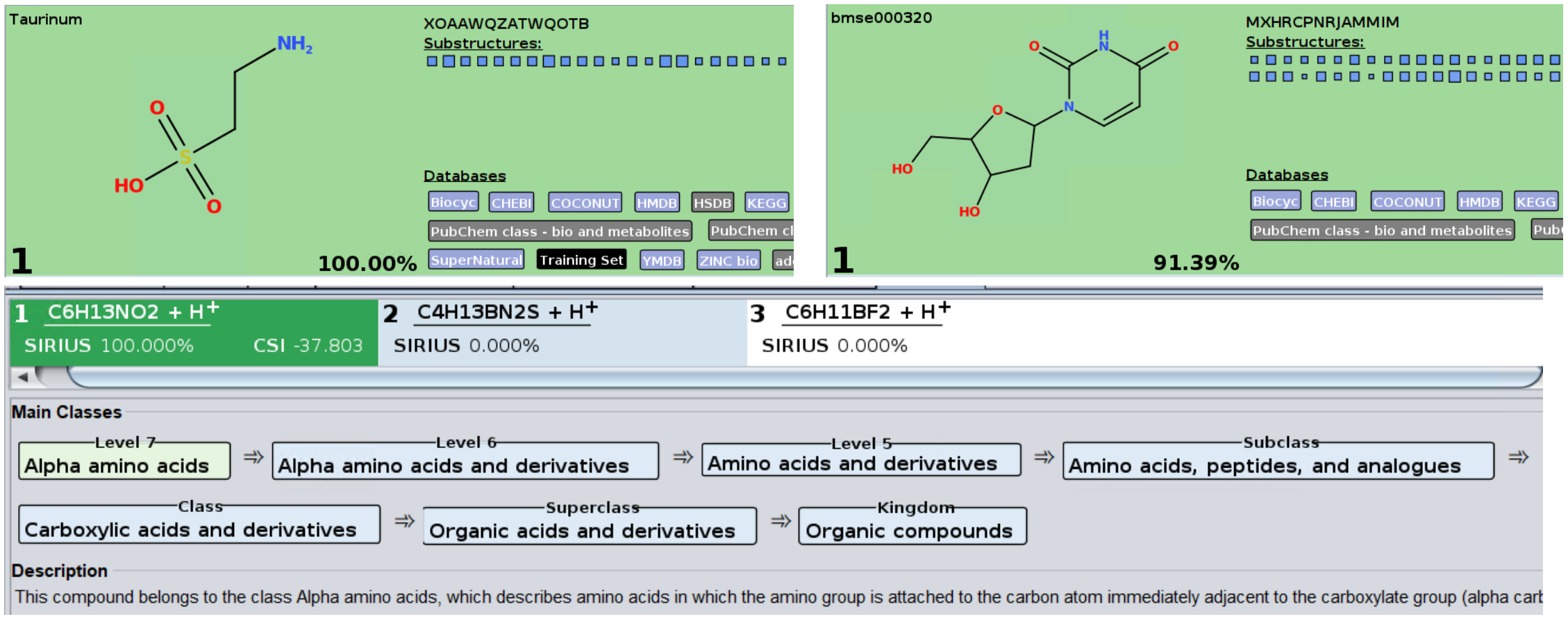


Figure 8: SIRIUS ID of two analytes from MS/MS spectrum collected from plasma extract (top). Along with a compound structure and ID the Databases in blue are links to that analyte in those databases. Using CANOPUS Compound Class Prediction[4] feature, class determination is made, and a description of the analyte is given (bottom) to aid in the ID understanding.

The statistical analysis showed separation of the two groups in a PCA plot (Fig 7). The entity list was comprised of 47 features that were significantly dysregulated in the mouse model. In MPP, the volcano plot highlights those features, and the clustering map shows each and how it trends in the different samples (Fig 7).

Finally, MS/MS spectra for analytes not identified from the custom PCDL were analyzed with SIRIUS software[3] (Fig 8). This second strategy uses an algorithm to suggest a structure based on the observed fragments and searches a comprehensive online structure database for analytes with matching or similar fragment patterns.

Out of the 47 significant features, 30 were identified with an overall score of 70 or more using PCDL and IDBrowser. Out of the remaining 17 unknowns, SIRIUS identified 5 features with a score of at least 82. When a feature was identified in SIRIUS confidence was given to the with the score, biologically related databases, and biological relationship from CANOPUS.

https://explore.agilent.com/asms

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Conclusions

Biological Insights are Found with this Complete Untargeted Metabolomics Workflow

- Metabolites extracted robustly using the Bravo Metabolomics platform and Captiva EMR-Lipid SPE plates
- Data collected using reproducible and transferable HILIC-Z chromatography and 6546 LC/Q-TOF for simultaneous isotopic fidelity, high resolution and extended dynamic range.
- Complete data analysis workflow using Profinder to extract features, MPP for statistical analysis, ID Browser and custom HILIC-Z PCDL, and SIRIUS to ID new unknowns.

References

[1] Sartain, M et al. Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform. Agilent Technologies. Application Note, 2020.

[2] Yannell, KE et al. Improvements to HILIC Robustness – a Targeted HILIC Metabolomics Method for Routine Analysis. ASMS, 2021.

[3] Dührkop, K. et al. SIRIUS4: a rapid tool for turning tandem mass spectra into metabolite structure information. Nat Methods, 16, 2019.

[4] Dührkop, K. et al. Systematic classification of unknown metabolites using high-resolution fragmentation mass spectra. Nature Biotechnology, 2020.

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