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Comprehensive workflow for targeted cell metabolomics using automated sample preparation, HILIC chromatography, LC/TQ, and a statistical analysis software suite

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Introduction

Targeted TQ Workflow for Metabolomics

Researchers aiming to understand the biological processes commonly use targeted LC/MS methods to quantify and profile the metabolome in large scale studies. For these metabolomic studies it is advantageous to have the most analytically-sensitive analysis and the most comprehensive list of metabolites to increase biological insights from the samples tested. Furthermore, the most effective LC/MS metabolomics workflows are simple to implement, reliable, reproducible, and provide deep biological insights with ease. Herein, is a new workflow for cellular metabolomics aimed at users with various LC/MS backgrounds by utilizes a Bravo automated sample prep and LC/MS for sensitive and reproducible studies (Fig. 1).

Figure 1: Instruments used for metabolomics workflow - Bravo Sample Prep Platform for reproducible sample prep (left), 1290 Infinity II Bio LC (center) has improved peak shape and detection limits for metal-sensitive metabolites, and 6495C LC/TQ (right) uses an ion funnel for low detection limits and low RSDs even with <1ms dwell times.

The Poroshell 120 HILIC-Z column was selected for retaining polar analytes and ionization in both positive and negative mode. It was paired with a 1290 Infinity II Bio LC (Fig. 1) which is coated with MP35N metal alloy to obtain sharp peak shapes and low detection limits for metal-sensitive analytes. A database of over 500 metabolites was built on the 6495C LC/TQ (Fig. 1), which was used to characterize the samples (Fig 5).

The data collected here characterized K562 cells (chronic myelogenous leukemia lymphoblasts) that underwent solid phase extraction (SPE) and reconstitution of metabolites (Fig. 2). The sample was spiked with 0.5-5 ppm of common intracellular analytes to create different groupings for a down stream statistical analysis that can be used to gain biological insights for metabolomics researchers. Analysis was performed with the methods described in Fig 3 and 4.

Experimental

Transferable Protocols for SPE Sample Prep and Reproducible HILIC Chromatography.

Figure 2: One million K562 cells/sample undergo cell lysis and quenching (left) followed by polar metabolite extraction on the Bravo (right).[1] Metabolites are selectively extracted, and lipids and proteins removed. Offline, the samples are dried and reconstituted in 80% ACN. For this study, a post-spike of a 40+ common intracellular analytes was used to create groupings.

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	

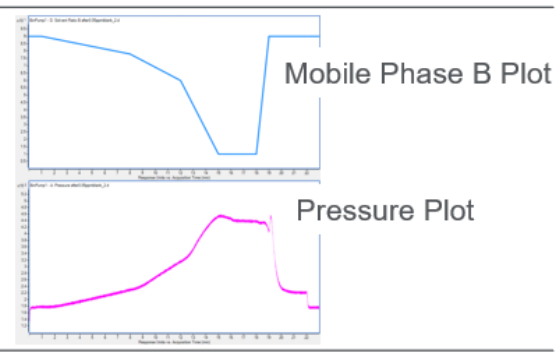

Figure 3: LC conditions produced retentions times with RSD <5% over an 11-day experiment and can be transferred to other systems.[2] *Detailed protocols for column prep and buffer prep ensure that the retention times are reproducible across different labs and skill levels. **0.5 mL/min flow rate used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

AJS Parameters	
Ion mode	Positive/Negative
Gas temperature	200 ºC
Drying gas flow	14 L/min
Nebulizer gas	50 psi
Sheath gas temperature	375 ºC
Sheath gas flow	12 L/min
Capillary voltage	(+)3000/(-)2500 V
Nozzle voltage	0 V

Figure 4: Source conditions for Agilent Jet Stream (AJS) coupled to a 6495C ion funnel LC/MS.

Experimental

Expansive dMRM LC/TQ Database for Fast Method Customization and Quick Turnaround to Biological Insights with Statistical Software Solutions

Using neat standards prepared in solvent and the MassHunter Optimizer software, a database of over 500 metabolites was created. **Analytes included are part of common metabolomics pathways such as glycolysis, TCA, amino acid metabolism, urea cycle, purine and pyrimidine metabolism.** Most of the metabolites have a minimum of 2 ion transitions for customization to the researcher's sample. **The database also contains retention times for the analytes. Given the reproducibility and transferability of the method[2] these can be used in a Dynamic MRM (dMRM) method for optimal sensitivity without limiting the number of compounds tested.** The transition selection and data analysis workflow is described in Fig 5.

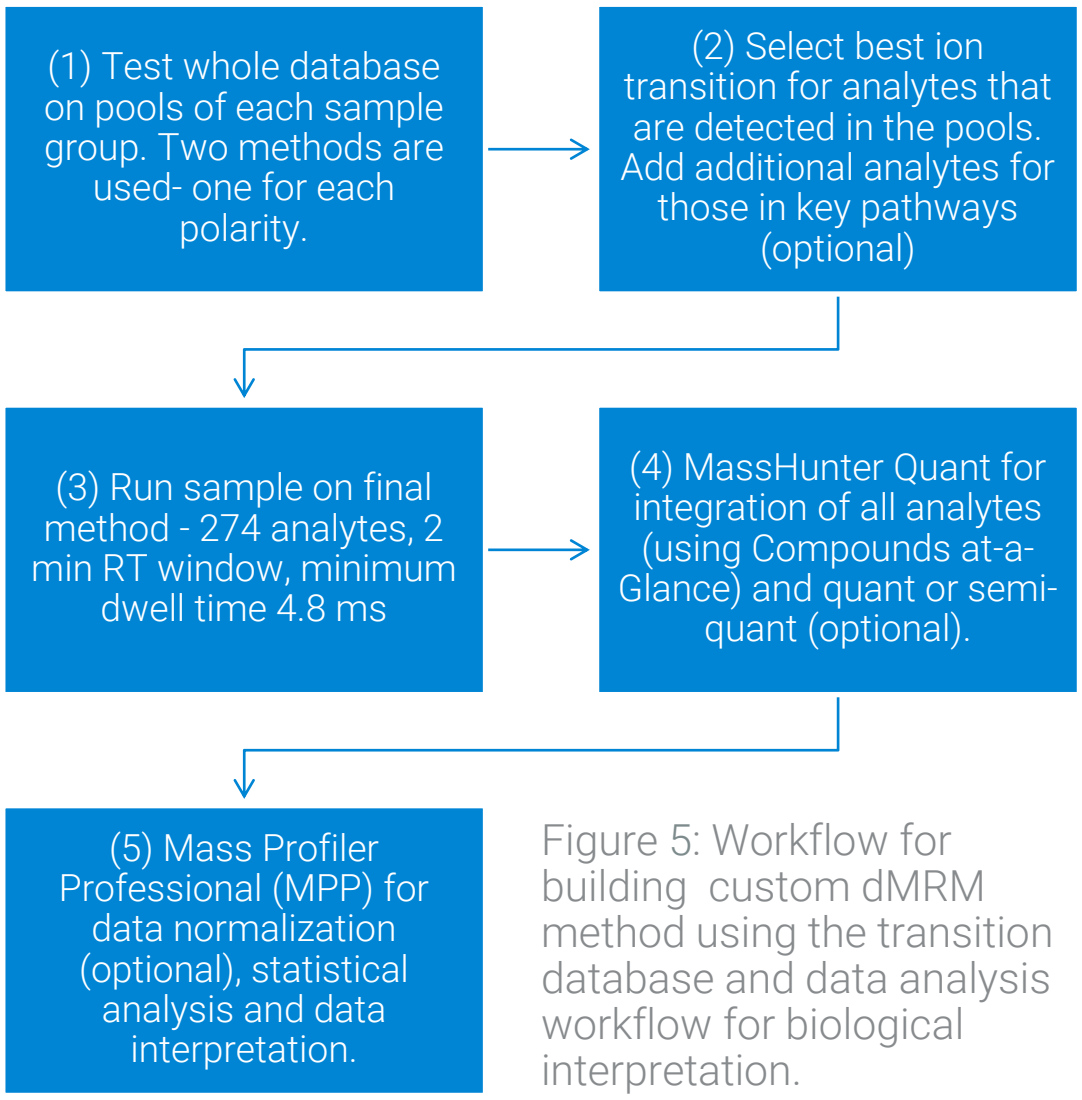

Figure 5: Workflow for building custom dMRM method using the transition database and data analysis workflow for biological interpretation.

The final method contained 274 analytes that were either detectable in the cell matrix and/or key to cellular metabolomic pathways. 5 sample groups were injected (n=6). To test the sensitivity of this method, a calibration curve 0.5-10,000 ng/mL was made for 13C-Phenylalanine.
For the MPP analysis, no normalization was needed. A statistical analysis wizard was employed to apply an ANOVA t-test (p=0.05) and fold change of 5.

Results and Discussion

Analytical sensitivity using the 6495C LC/TQ can detect cellular metabolites from various compound classes with ease.

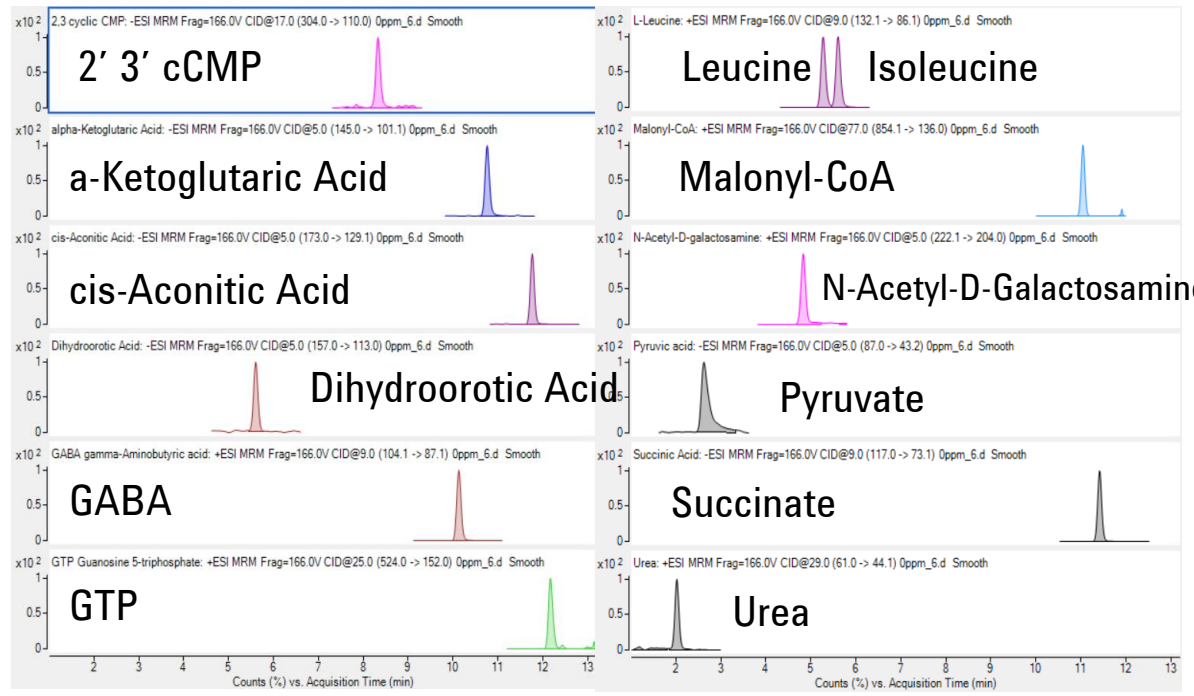

Figure 6: Chromatograms showing the peak shape and separation of metabolites in cell matrix with no spike in. Using the HILIC-Z column and standardized column and buffer prep, these retention times and peaks are reproducible and transferable making a dMRM method possible.

The whole database was tested on a pooled cell extract. This identified the 250+ analytes that can be detected at <1ms dwell time on the system. When combining that list with additional analytes to round out certain pathways the total list was 274 metabolites. When using a 2 min RT window the minimum dwell time was <5 ms. This method has the capability of measuring down to fmol levels as shown by the calibration curve in Fig 8. **The speed of the 6495C allows reproducible and sensitive measurements even when low dwell times are used. This means that each injection can hold hundreds of analytes and give researchers more information about the sample.**
MassHunter Quantitative analysis was used to integrate all the transitions in the dataset. This task was fast because the software tool Compounds at-a-Glance was used which displays compounds and samples in a grid format and allows integration editing for efficient review. If profiling is not the only goal but also quantitation or semi-quant a calibration curve can be used here to calculate concentration of analytes. A .CSV file export from MassHunter Quant moves the analytes over to MPP for statistical analysis.
In MPP a statistical wizard moves the data through the program to apply normalization, baselining, variance testing, and statistical tests. A shortened list of <60 analytes were altered. All the spike analytes were in the list. The additional analytes that are altered could be removed if normalization is applied with a pooled QC.

Results and Discussion

Database and Database Browser Provides Fast and Convenient Way to Build and Customize a Method and Gain Biological Insights Fast.

The majority of unaltered features in the sample have a RSD of <20% (n = 30, not normalized). The PCA plot in Fig 7 also shows tight groupings of replicate injections of the spiked samples. Normalization is recommended for longer experiments and it is recommended to use signal from a pooled matrix QC injected every 5-10 samples for normalization.

MPP provides several useful plots to interrogate the trends in a sample. A heat map is shown in Fig 7 and box-and-whisker plots, volcano plots, hierarchical clustering, and violin plots can also be useful for this type of research.

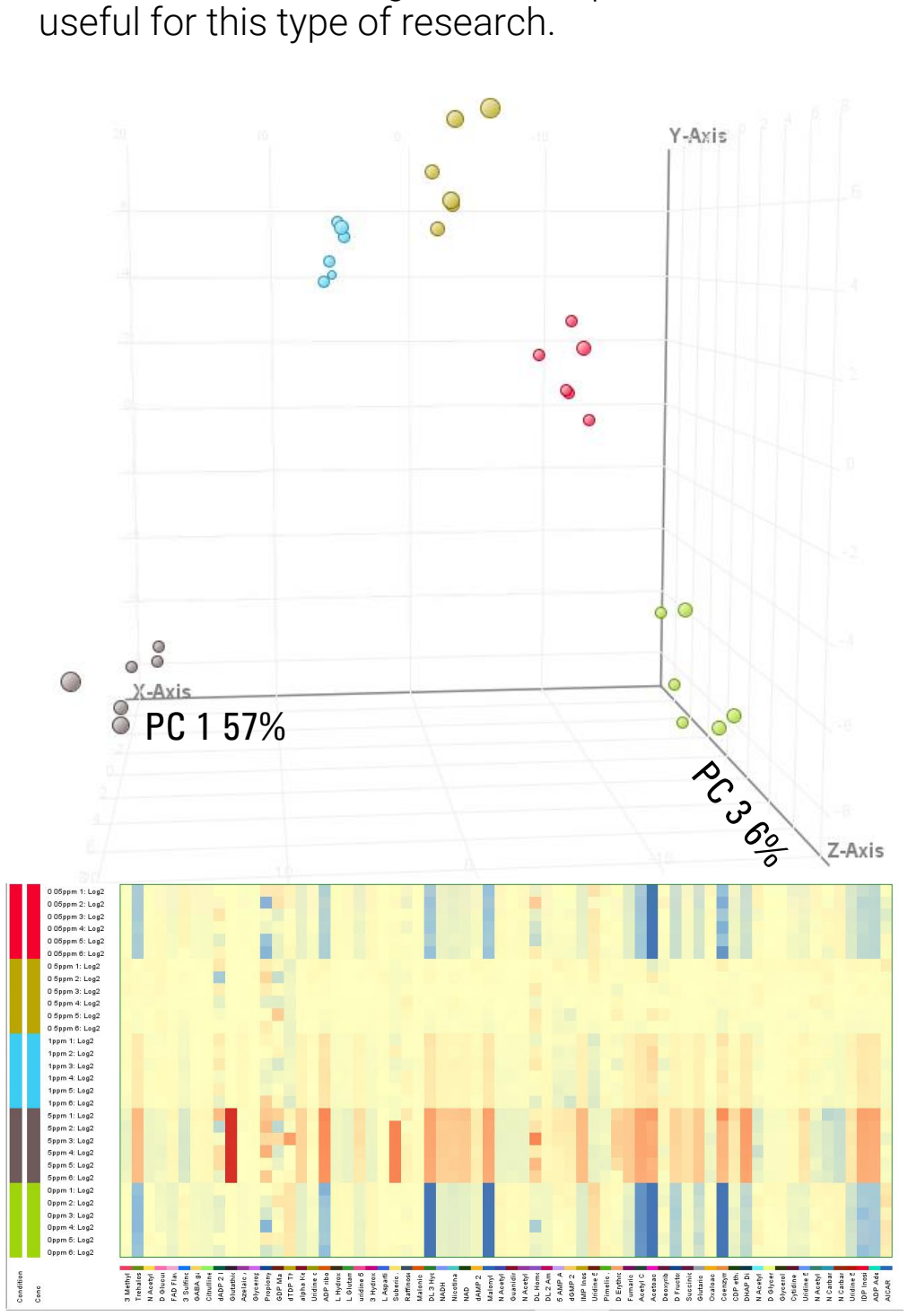

Figure 7: PCA plot (top) shows clear groupings of the 5 cell sample spikes. Green no spike, red 0.05 ppm, yellow 0.5ppm, blue 1ppm, and black 5ppm. A portion of the heat map shows features that are altering in the spike samples which correspond with the analytes in the spiked mixture.

Data Analysis Workflow Is Simple and Produces Clear Lists of Analytes Changing in Samples.

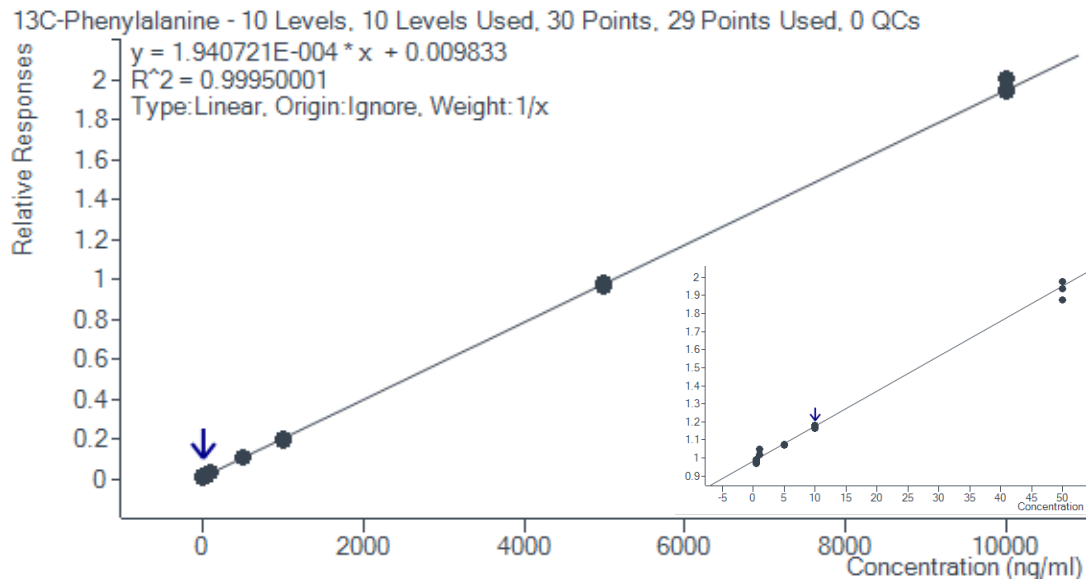

Figure 8: Calibration curve for 13C- Phenylalanine from 0.5 – 10,000 ng/mL normalized to endogenous phenylalanine (n=3). Results are linear and the method is sensitive beyond 6 femtomole on column in cell matrix.

Conclusions

Jump-start biological research projects with a comprehensive workflow that includes sample prep, transferable chromatography[2] and extensive dMRM database.

- Polar metabolites were effectively extracted from K562 cells using a robust Bravo-automated protocol.
- A comprehensive dMRM method can be built using the custom database of ion transitions and retention times for over 500 analytes.
- The 6495C LC/TQ provided needed speed and analytical sensitivity to detect analytes with <5ms dwell times. Limits of quantitation vary but are in the fmol range in matrix for the isotopically-labeled standards.
- Data analysis in MPP can help researchers find trends in the data quickly with or without quant.

References

[1] Van de Bittner, GC et al. A Comprehensive Workflow for Routine, Automated, Metabolite + Lipid Analysis of Mammalian Cells. Metabolomics Conference Poster, 2020, #74.

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

https://explore.agilent.com/asms

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