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Automated Dual Metabolite+Lipid Cell Sample Prep as a Component of an LC/HRMS-based Workflow to Elucidate the Molecular Response to Drug Treatment

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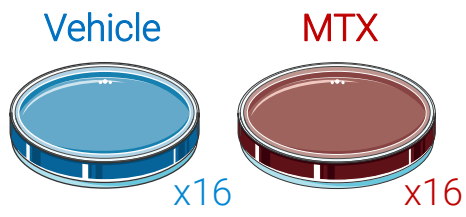
Introduction

Practical challenges exist for mammalian cell sample preparation workflows for polar metabolite and lipid extraction. To overcome these challenges, we applied a novel cellular extraction method that lyses mammalian cells and quenches metabolism under room temperature conditions and developed an automated method that sequentially extracts both polar metabolites and lipids from a single sample of harvested cells. Here we demonstrate comprehensive untargeted metabolomics and lipidomics workflows from sample prep to data analysis within the context of cancer cell biology. Metabolite and lipid extracts were directly analyzed with LC/Q-TOF and a suite of discovery-based MS software to elucidate the molecular response to perturbations in an acute myeloid leukemia cell line in response to treatment with the drug methotrexate (MTX).

Experimental

Sample Preparation

Acute myeloid leukemia K562 cells were cultured in supplemented RPMI medium and treated with 1 µM methotrexate or saline for 24 hours. Upon harvesting, 1 million K562 cells per sample (n=32) were lysed and quenched at room temperature with 50% trifluoroethanol in water.



In a separate experiment, representative 13C-metabolites and 2H-lipids were used to estimate sample prep recoveries. A 13C-yeast extract combined with 13C-standards comprised the polar metabolite spike-in. The Avanti UltimateSPLASH ONE mixture was used as the 2H-lipid spike-in.

The dual metabolite + lipid extraction process was automated using an Agilent Bravo Liquid Handling Platform using an in-house developed automated protocol.

LC/MS and Software Analysis

Lipids and polar metabolite extracts were separated with (+/-) RP-LC and (-) ion-pairing RP-LC, respectively, and eluents analyzed with an Agilent 6546 LC/Q-TOF.

Both studies were achieved with a combination of Agilent MassHunter Quantitative Analysis, Profinder, Lipid Annotator, and MPP software.

Experimental

Overview of Dual Metabolite+Lipid Cell Workflow

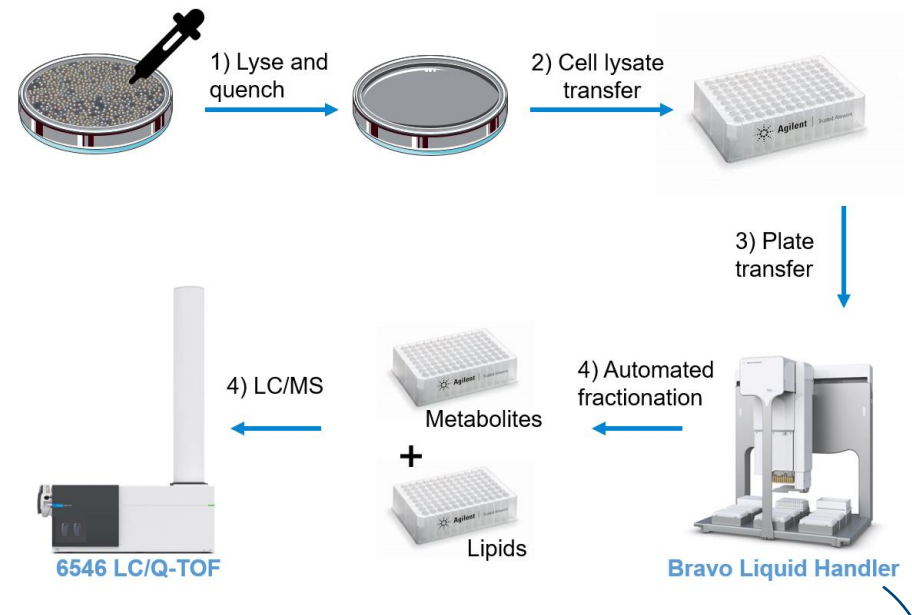
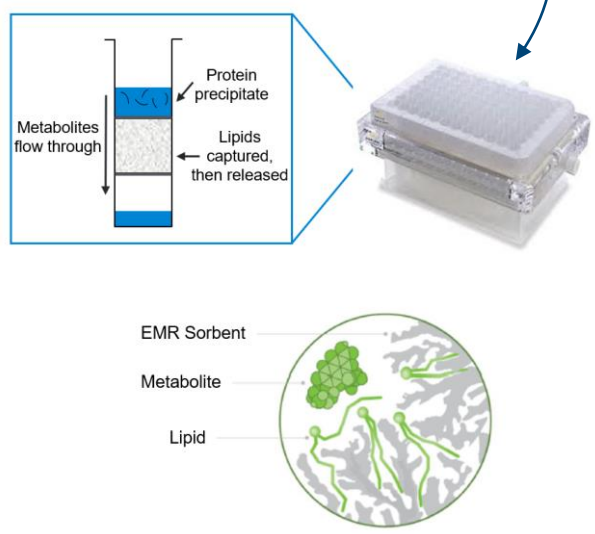


Figure 1. Overview of workflow, from sample preparation to LC/MS analysis.

Figure 2. The Captiva EMR-lipid plate filters proteins, captures lipids, and elutes metabolites.[1] After metabolite elution and a plate wash, lipids are eluted by switching to a lipid-elution solvent.[2]



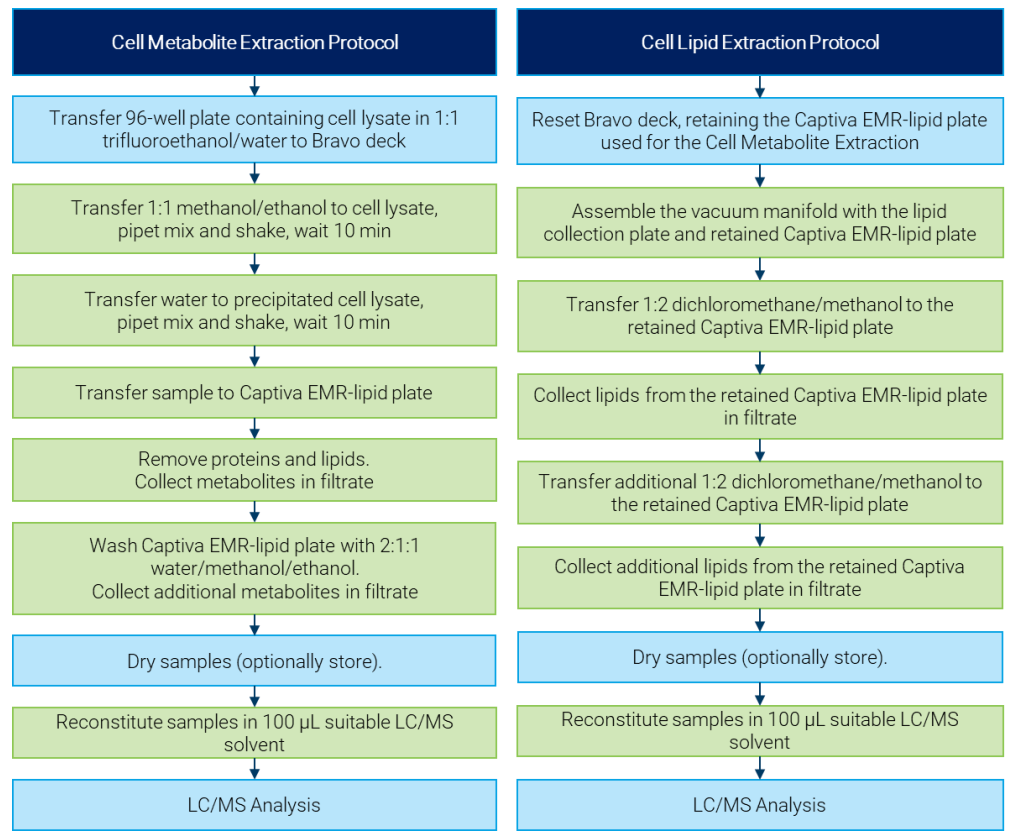
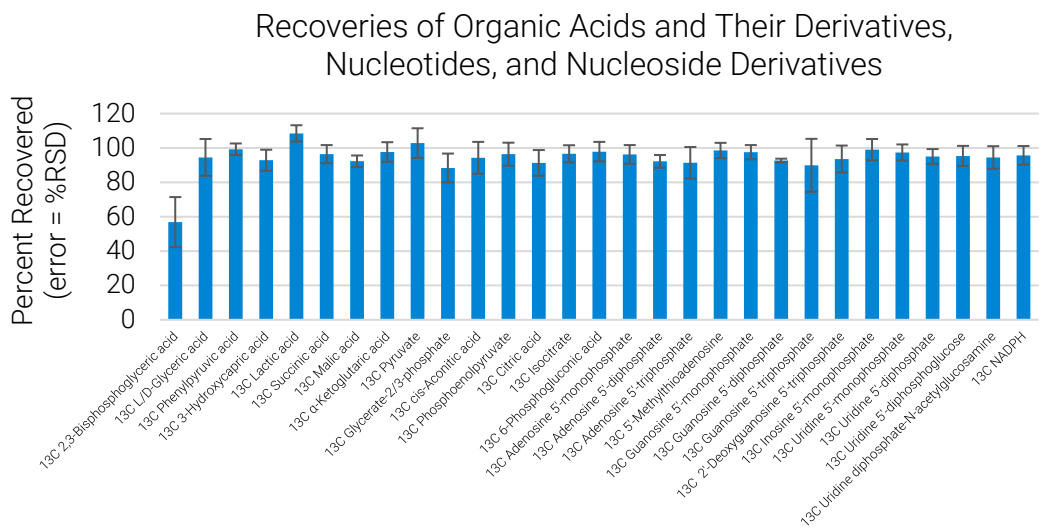


Figure 3. Overview of workflow steps for dual metabolite+lipid workflow. Green steps are automated on Bravo Liquid Handler and blue steps are performed manually. Cell quenching and lysis is performed manually prior to starting the automated workflow.

Method Provides Good Metabolite and Lipid Recoveries and Excellent Percent Relative Standard Deviations

Preliminary polar metabolite and lipid recoveries were obtained with pre/post spike-ins of labeled compounds.



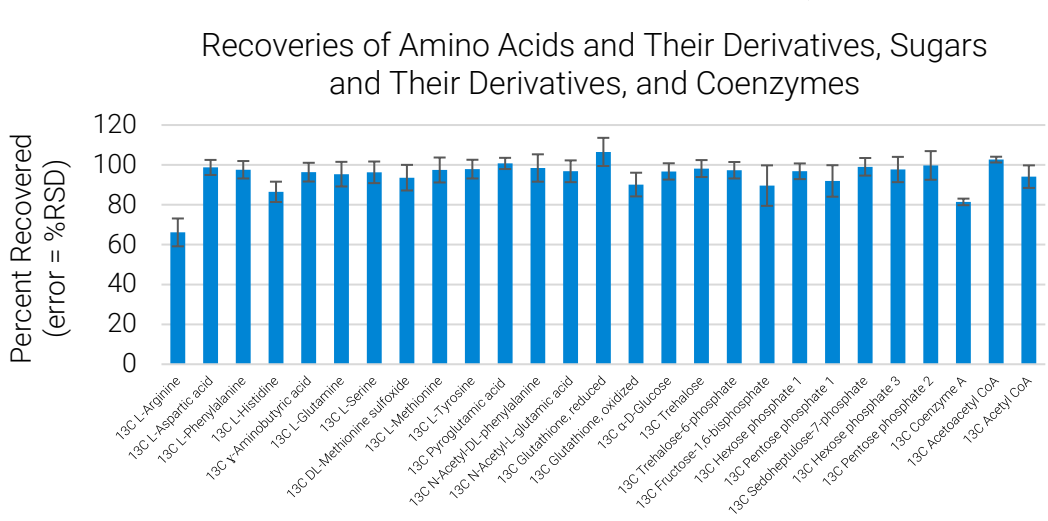


Figure 4. Recoveries across all major polar metabolite classes were excellent with 87% of compounds having a recovery between 90% and 110%. The average %RSD across all polar metabolites was exceptional at 5.89%, with the lowest %RSD at 1.11% and with 93% of compounds having a %RSD <10%.

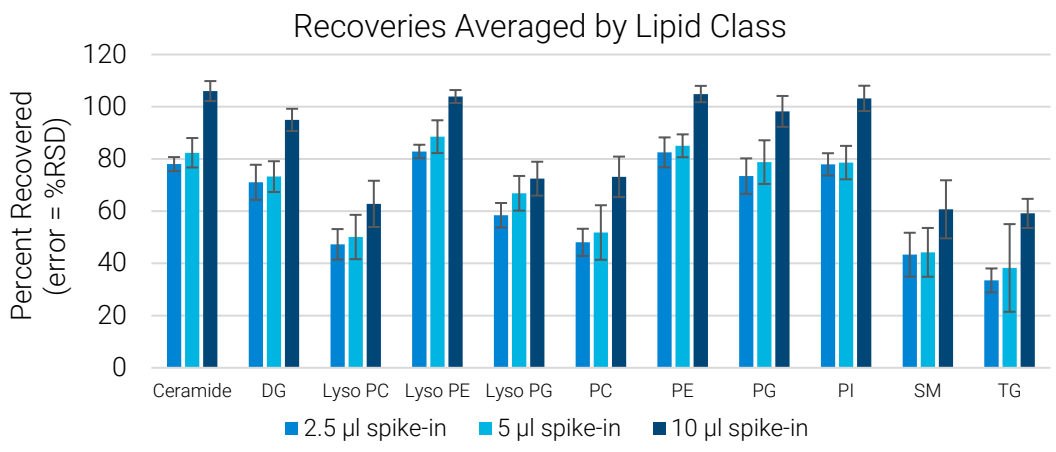


Figure 5. Lipid recoveries were analyzed across three spike-in volumes and averaged by lipid class. Recoveries are lower than the polar metabolite recoveries, which we found acceptable for LC/MS due to the generally higher abundance and ionization of lipids. The average %RSD across all lipids and all spike-in volumes was excellent at 6.38%. Average %RSDs for each lipid class are <10% when all spike-in volumes are combined, indicating good %RSD performance across lipid abundances.

Metabolomics Data Analysis Workflow

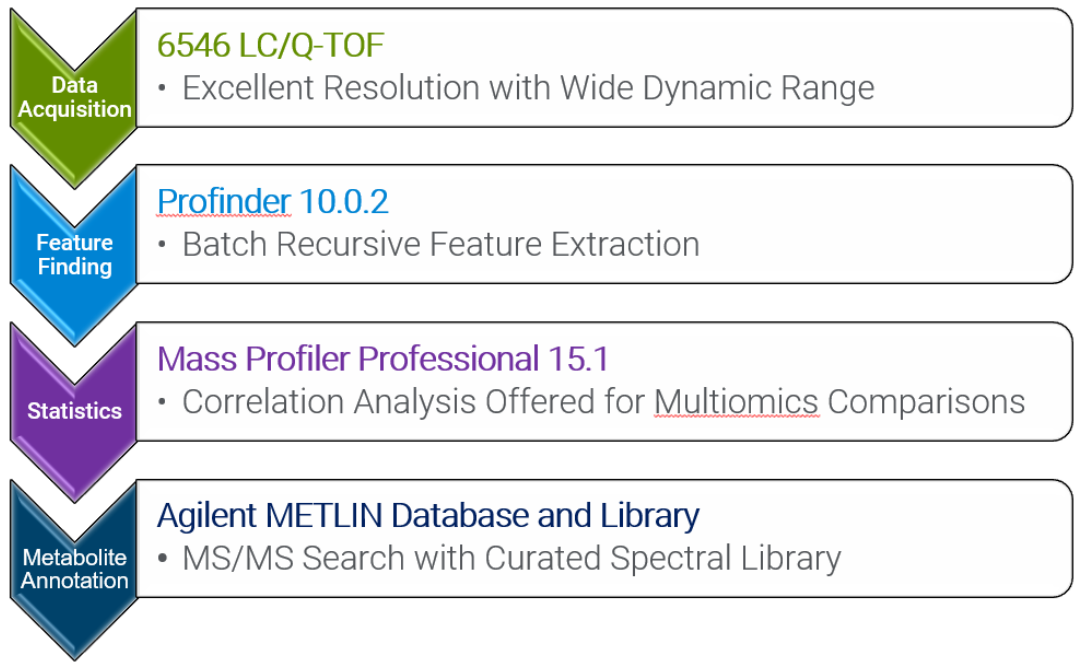


Figure 6. Major steps and corresponding hardware/software with key features are detailed.

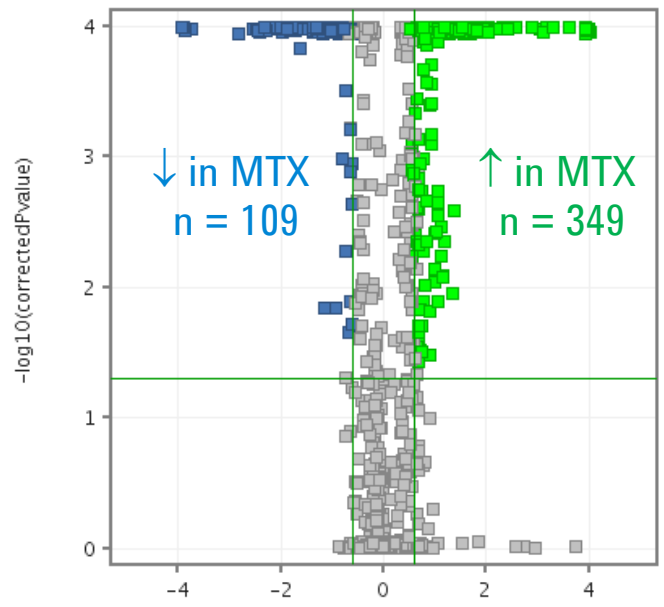


Figure 7. Untargeted batch recursive feature extraction with Profinder software resulted in 4700 compounds. Shown is a volcano plot with cutoffs of 1.5-fold change and *p*-value <0.05 resulting in 458 significant features.

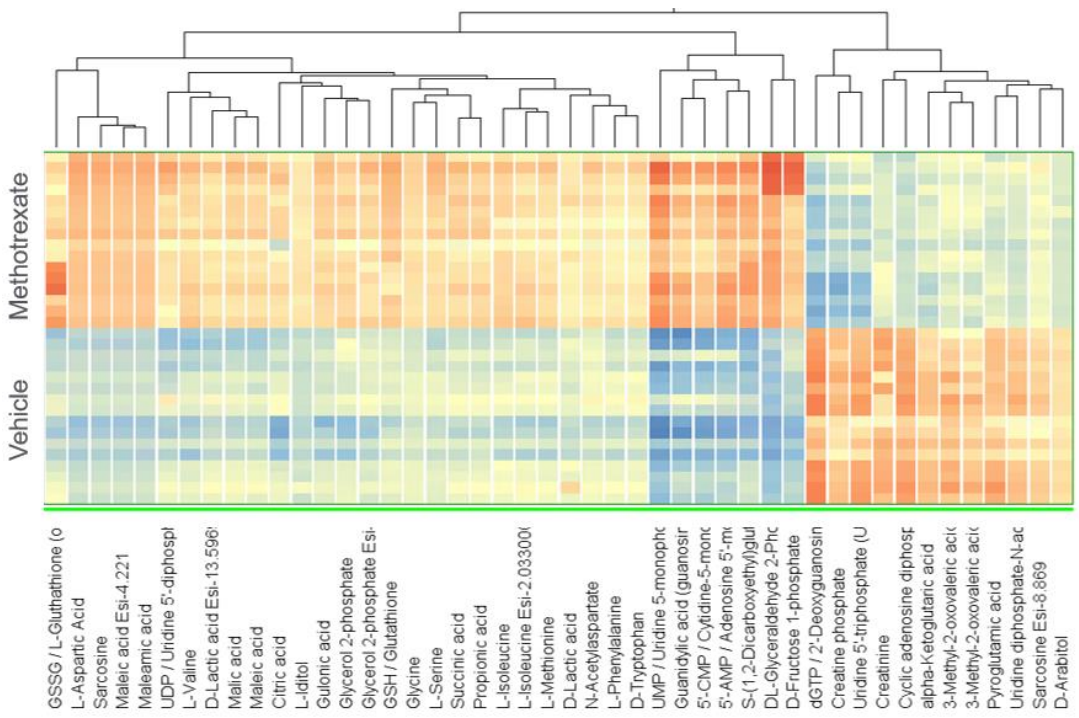


Figure 8. MS/MS data corresponding to significant features were searched against the Agilent METLIN spectral library. Clustering analysis of 48 significant annotated metabolites is shown. Similar to Funk et al.[3], purine and pyrimidine biosynthesis appeared to be largely affected, followed by amino acid and TCA metabolism.

Results and Discussion

Lipidomics Data Analysis Workflow

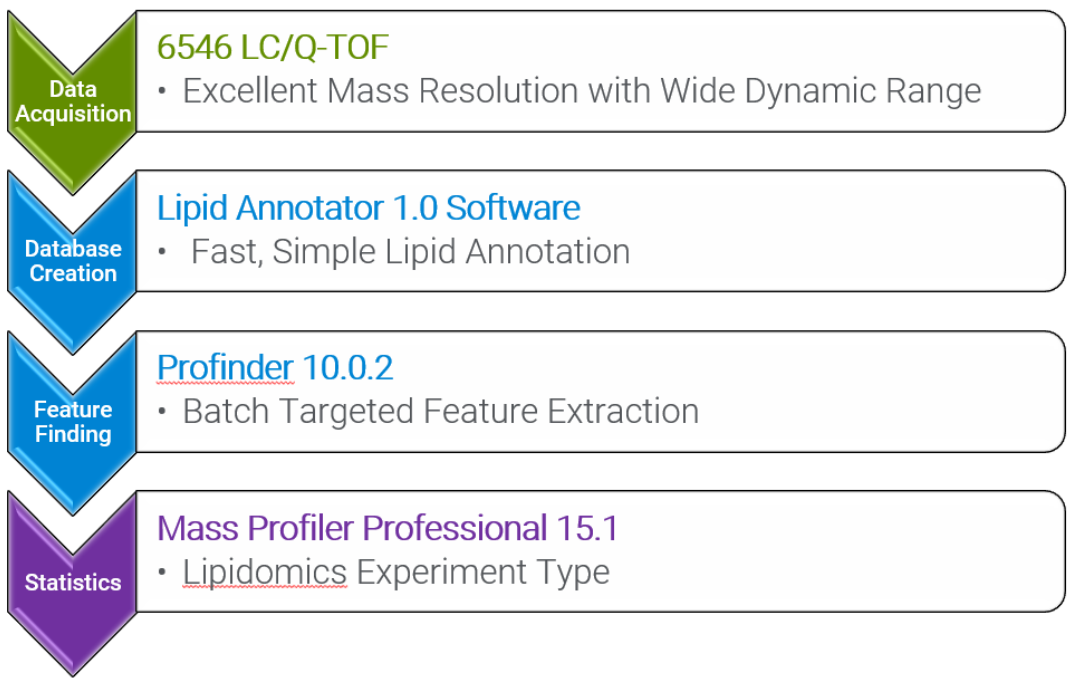


Figure 9. Major steps and corresponding hardware/software with key features are detailed.

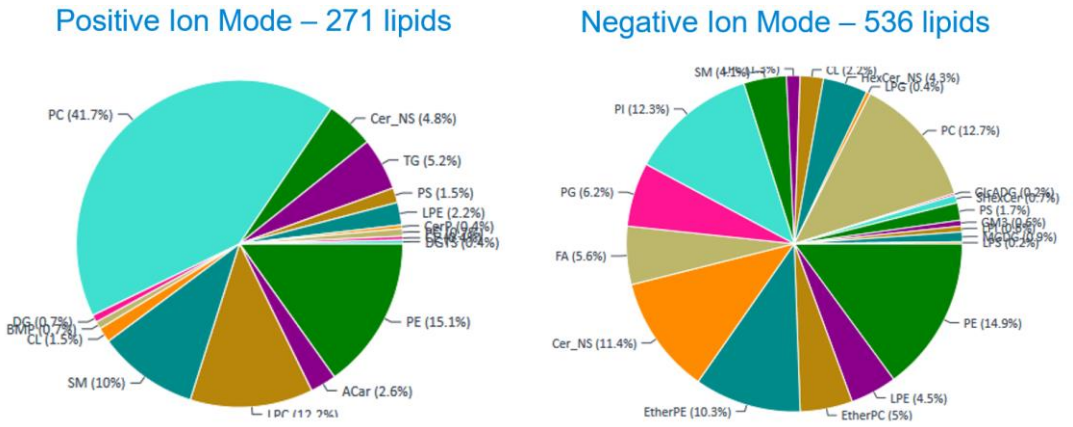


Figure 10. Agilent Lipid Annotator software results from 2 batches of 5 Iterative MS/MS datafiles acquired on pooled K562 cellular lipid extracts prepared with the dual automated workflow. The databases generated from the annotations were used to drive the remainder of the lipid profiling workflow.

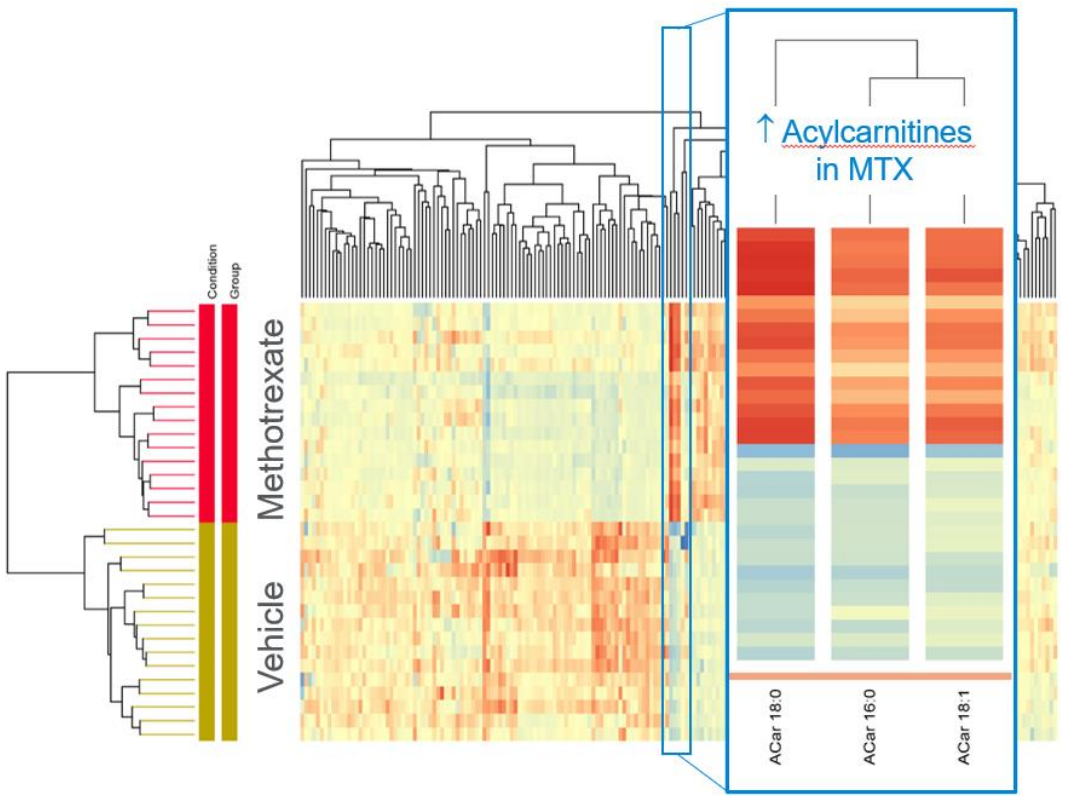


Figure 11. Unsupervised hierarchical clustering in MPP software revealed cellular acylcarnitine (Acar) levels increase with MTX treatment, a novel finding compared to a previous approach.[3]

Intra-Sample Multi-Omics Analysis

Isolation of metabolites and lipids from the same physical samples is ideal for direction comparison with correlation analysis.

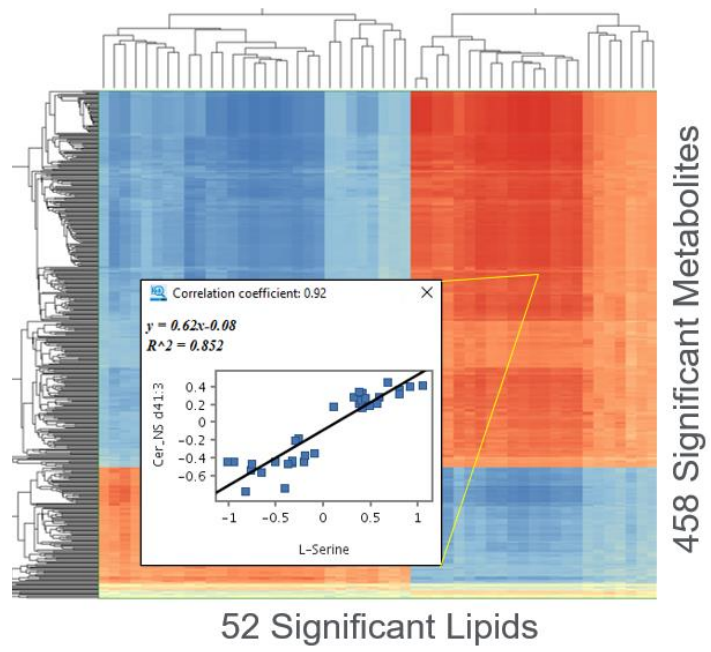


Figure 12. Multi-omics correlation analysis in MPP software. The zoomed inset shows correlation across sample replicates for highly correlated L-serine and a ceramide (Cer_NS) species (corr coeff=0.92, R^2=0.85), that both increase with MTX treatment. L-serine was also positively correlated with multiple ceramides. This link may be explained as MTX impacting ceramide biosynthesis through L-serine.

Conclusions

A Combined Cell Metabolite + Lipid Workflow Enabled:

- Stable, room temperature processing of cell samples
- Automation-compatible metabolite and lipid fractionation and collection
- Intra-sample multi-omics analysis

LC/MS Analysis Revealed Methotrexate Treatment Caused:

- Increased ceramides and acylcarnitines, and decreased short-chain phosphatidylinositols
- Buildup of metabolite intermediates in pyrimidine and purine biosynthesis

References

[1]Spivia, W, et al. Automated Metabolite Extraction for Plasma using thAgilent Bravo Platform. Agilent Application Note 5994-0685EN, 2019e.

[2]Apffel, A, et al. A Novel Solid Phase Extraction Sample Preparation Method for Lipidomic Analysis of Human Plasma… Metabolites, 2021, 11(5): 294.

[3]Funk, R.S. et al. Metabolomic Profiling to Identify Molecular Biomarkers of Cellular Response to Methotrexate In Vitro. Clin Transl Sci., 2020 January; 13(1): 137-146.

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