Application Note

Metabolomics

Agilent

Trusted Answers

An End-to-End Targeted Metabolomics Workflow

Author

Karen E. Yannell, PhD
Cate Simmermaker
Genevieve Van de Bittner, PhD
Daniel Cuthbertson, PhD,
Agilent Technologies, Inc.

Abstract

The metabolome refers to all the small molecules produced by cells or an organism during metabolism. As such, metabolomics data can be used as a direct functional readout of metabolic activity and physiological status. Targeted metabolomics aims to routinely detect and quantify a predefined group of metabolites likely to be involved in biological processes of interest. This application note presents a robust workflow that combines sample preparation and instrumental analysis solutions for targeted metabolomics, and can be applied to plasma and mammalian cell sample types. Automated sample preparation was achieved by combining the Agilent Bravo Metabolomics Sample Prep platform and Agilent Captiva EMR–Lipid plates. Metabolite separation was performed using the Agilent 1290 Infinity II Bio LC ultra-high performance liquid chromatography system coupled with an Agilent InfinityLab Poroshell 120 HILIC-Z column. Metabolite detection were performed using the Agilent 6495 Triple Quadrupole LC/MS system coupled with a custom database of 500 metabolites made using the Agilent MassHunter Optimizer software. The dynamic multiple reaction monitoring (dMRM) method was used with different numbers of ion transitions but can hold one transition for each of the 500 metabolites and can operate reproducibly at a 1 ms dwell time. Additionally, data analysis was performed using the Agilent MassHunter Quantitative Analysis 10 and Mass Profiler Professional (MPP) software. The results presented here demonstrate that this method can be used to efficiently separate metabolites from multiple compound classes in a reproducible way. Moreover, the 6495 Triple Quadrupole LC/MS allows for the detection of metabolites with great sensitivity, even at low dwell times. In summary, this is a reproducible and easy-to-use method that can be customized to fit specific needs and is suited to researchers with varying metabolomics expertise.

Introduction

Cells produce, transform, and consume small molecules (< 1500 Da) also known as metabolites; collectively, these are the metabolome. These metabolites are crucial as they reflect an organism's phenotype. They include molecules that are synthesized during metabolism, such as glucose, cholesterol, ATP, lipids, amine neurotransmitters, amino acids, organic acids, and steroids.[1] Metabolomics research aims to measure these small molecules and find correlations with different physiological states.[2] Metabolomics methodologies broadly fall into two categories: untargeted and targeted analyses.

Untargeted metabolomics encompasses a comprehensive analysis of all the measurable analytes in a sample, including those which are unknown compounds. In contrast, targeted metabolomics measures defined groups of annotated metabolites.[2] As a result, untargeted analysis is typically used for metabolite discovery, while targeted metabolomics is used to provide relative or absolute quantification and validation of specific metabolites of interest.

An end-to-end targeted metabolomics workflow involves sample preparation, separation, detection, and statistical analysis of the metabolites and, thus, requires a combination of different analytical techniques. Agilent offers a full suite

Figure 1: Agilent solutions for targeted metabolomics.

of hardware, consumables, and software for every step of the workflow (Figure 1). Moreover, all protocols, methods and databases are already packaged and ready for quick implementation in any lab.

The workflow presented here combines automated sample preparation, hydrophilic interaction chromatography (HILIC), and triple quadrupole mass spectrometry (LC/TQ) to achieve targeted metabolite detection. As a result, researchers with varying levels of metabolomics expertise can obtain sensitive and reproducible results from various sample types.

Methods

Sample preparation

Sample preparation was completed using the Bravo Metabolomics Sample Prep Platform in combination with Agilent Captiva EMR–Lipid solid phase extraction plates (Figure 2A and B). For cell sample preparation, supplementary Dual Metabolite + Lipid Cell Sample Preparation VWorks protocols are also required.[3] Automation of the sample preparation process using the Bravo platform improves ease-of-use, reduces hands-on time and increases reproducibility, in part through a reduction in user errors. The Captiva EMR-Lipid technology removes lipids based on a combination of size exclusion and hydrophobic interaction. Effective depletion of lipids with long hydrocarbon chains minimizes ion suppression of target polar metabolites and improves method reliability and ruggedness.[4] This step is also essential to reproducibly elute polar metabolites and enhance chromatographic robustness.

Metabolites were extracted from 20 µL of pooled bovine plasma (BioIVT) and one million K562 chronic myeloid leukemia-derived cells following protocols described in previous application notes.[3,5] Cells were first isolated from their medium, washed with PBS, lysed, and their metabolism was quenched before the cell lysate was added to a 96-well plate. For both sample types, polar metabolite extraction was automatically performed using the Bravo platform using the general protocol shown in Figure 2C. The workflow delivers a plate with polar metabolite extracts ready to be dried and reconstituted for LC/MS analysis.

Separation

The metabolites extracted in the previous step were separated using the 1290 Infinity II Bio LC system (Figure 3A). This ultrahigh performance liquid chromatography (UHPLC) system

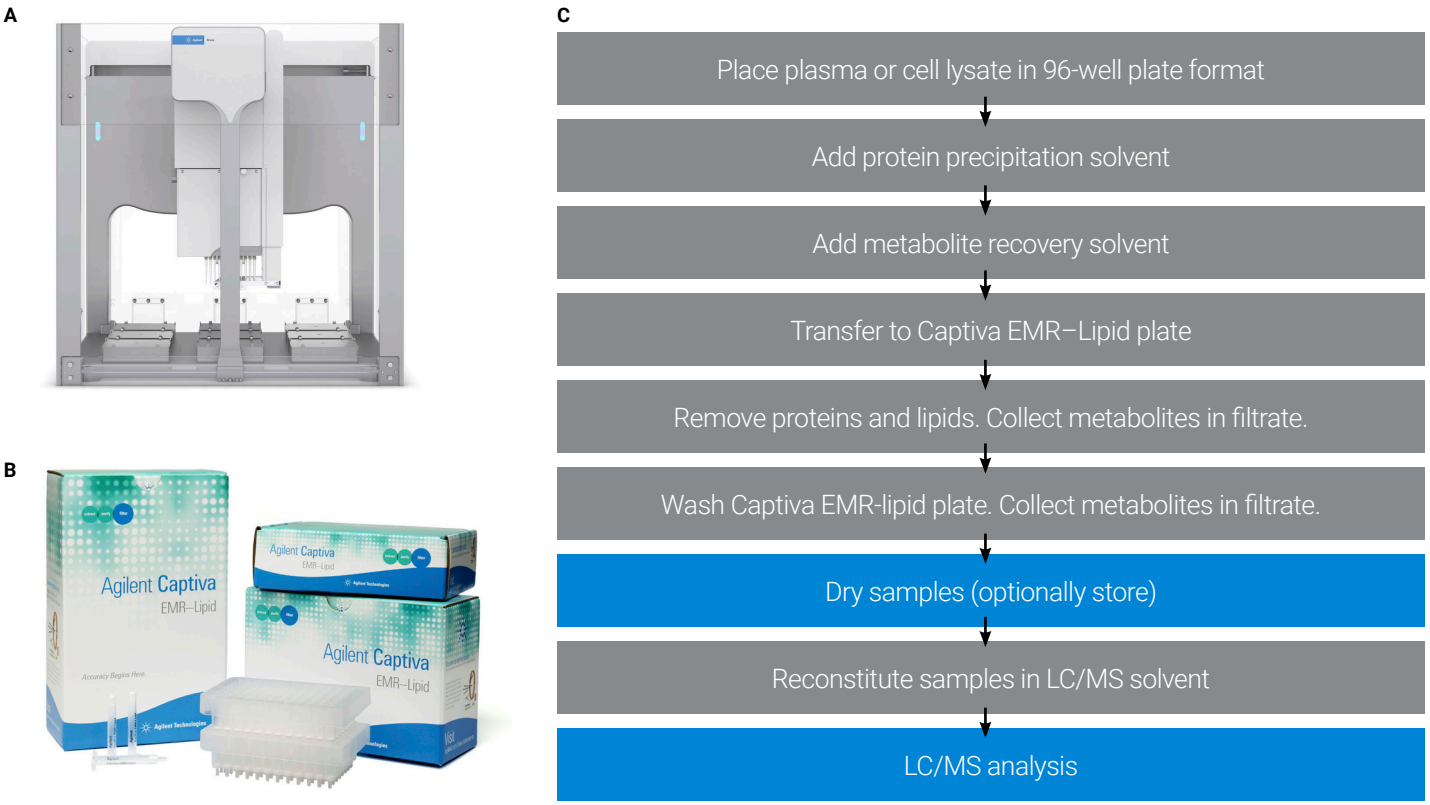

Figure 2: Agilent solutions for automated sample preparation for targeted metabolomics research. **A**. Bravo Metabolomics Sample Prep platform. **B.** Captiva EMR–Lipid solid phase extraction plates. **C.** General polar metabolite extraction protocol.

A

B

LC Conditions	
Column	Agilent InfinityLab Poroshell 120 HILIC-Z, 2.1 x 150 mm 2.7 µm, PN 683775-92
Column temperature	15 °C
Injection volume	3 µL
Autosampler temp	5 °C
Needle wash	Standard Wash, 10 sec, IPA:ACN:H_2O 1:1:1
Mobile phase	A = 20 mM ammonium acetate, pH 9.3 + 5 µM medronic acid in water B = pure ACN
Flow rate	0.400 mL/min
Gradient program	Time %B 0.00 90 1.00 90 8.00 78 12.00 60 15.00 10 18.00 10 19.00* 90 23 90
Total run time	24 min

Figure 3: A. Agilent 1290 Infinity II Bio LC system. **B.** Protocol used for polar metabolite separation.* During re-equilibration a 0.5 mL/min flow rate was used.

is coated with MP35N metal alloy to obtain reproducible peaks and low detection limits for metal-sensitive analytes. The system was coupled with a InfinityLab Poroshell 120 HILIC-Z column to provide good retention of polar analytes while permitting ionization in both positive and negative modes.

The conditions used are detailed in Figure 3B. A Poroshell 120 HILIC-Z column (2.1 x 150 mm, 2.7 µm) was flushed with a standardized phosphorylation procedure and equilibrated for one hour with analysis buffers (30:70 A:B). The buffer system contained 20 mM ammonium acetate, pH 9.3 + 5 uM medronic acid in water (mobile phase A) and pure acetonitrile (mobile phase B). A nonlinear gradient was used for analysis (elution times span from 0.5 - 16 min over the gradient). Each injection lasted 24 min including re-equilibration time. This method and associated protocols were tested on multiple column lots, in different labs, and with users of different skill levels. Such optimization and testing ensures reproducible retention times and transferability to different labs.

Detection and Analysis

Equipment: Metabolite detection was performed using the 6495 Triple Quadrupole LC/MS (LC/TQ) system (Figure 4A) – a powerful and reliable mass spectrometer that produces reproducible and sensitive data at low dwell times (0.5 ms).

A

B

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

Figure 4: A. Agilent 6495C Triple Quadrupole LC/MS. **B.** Parameters used for the AJS. iFunnel positive high pressure RF 150 V, positive low pressure RF 60, negative high pressure RF 90 V, negative low pressure RF 60.

A third-generation ion funnel collects and focuses stray ions, while pumping out neutrals. The Agilent Jet Stream (AJS) achieves highly efficient desolvation and produces five times more ions than standard electrospray technology thanks to thermal focusing and heat transfer. The ion source conditions used for the AJS are summarized in Figure 4B.

Database curation: The dynamic multiple reaction monitoring (dMRM) method was used with various ion transitions.[6] The dwell times were reproducible down to the lowest tested value (1 ms). The MassHunter Optimizer software was used to automatically build a database of ion transitions for over 500 analytes. Neat standards were used to optimize the MRM transitions for each analyte. These transitions were verified, curated, and assigned to retention times. When possible, ion transitions in positive and negative ion mode were added and at least two transitions for each ion mode were included.

Method customization: Database implementation can be customized for each experiment to give researchers the flexibility to profile metabolites or build quantitative methods. There are four potential workflows: 1) profiling on all analytes, 2) profiling on specific analytes known to be present in the sample, 3) profiling on pathways and 4) quantitative or semi-quantitative method using stable isotope products from Cambridge Isotope Laboratories.[7] The second workflow was used in this application note (Figure 5).

Software: The MassHunter 10 Acquisition software was used to control the LC/TQ instrument. Quantitation and integration of all analytes was performed with the MassHunter Quantitative Analysis 10 software. Mass Profiler Professional (MPP) was used to perform statistics on the data. This software uses automation and wizards to simplify the analysis.

Results and discussion

This workflow resulted in the identification of 266 and 274 metabolites in the plasma and cell samples, respectively. The curated database contains over 500 analytes providing good coverage of amino acids, coenzymes, tricarboxylic acid (TCA) cycle, and glycolysis pathway analytes. This allows the researcher to confidently probe the fundamental pathways of energy metabolism.

Reproducible peaks for various analyte classes

Metabolites with a range of different chemistries were efficiently and reproducibly separated using the 1290 Infinity II Bio LC system coupled with HILIC-Z column (Figure 6). The HILIC method allowed for the reproducible separation of several polar isomeric compounds, such as leucine and isoleucine, without the use of ion-pairing reagents (Figure 6B). Ion-pairing reagents – used in traditional C18 methods to retain small metabolites – introduce a background of positively-charged ions to the LC/MS system, preventing analysis in positive ion mode. Without need for ion-pairing reagents, HILIC method retains metabolites and other small molecules allowing researchers to increase the metabolome

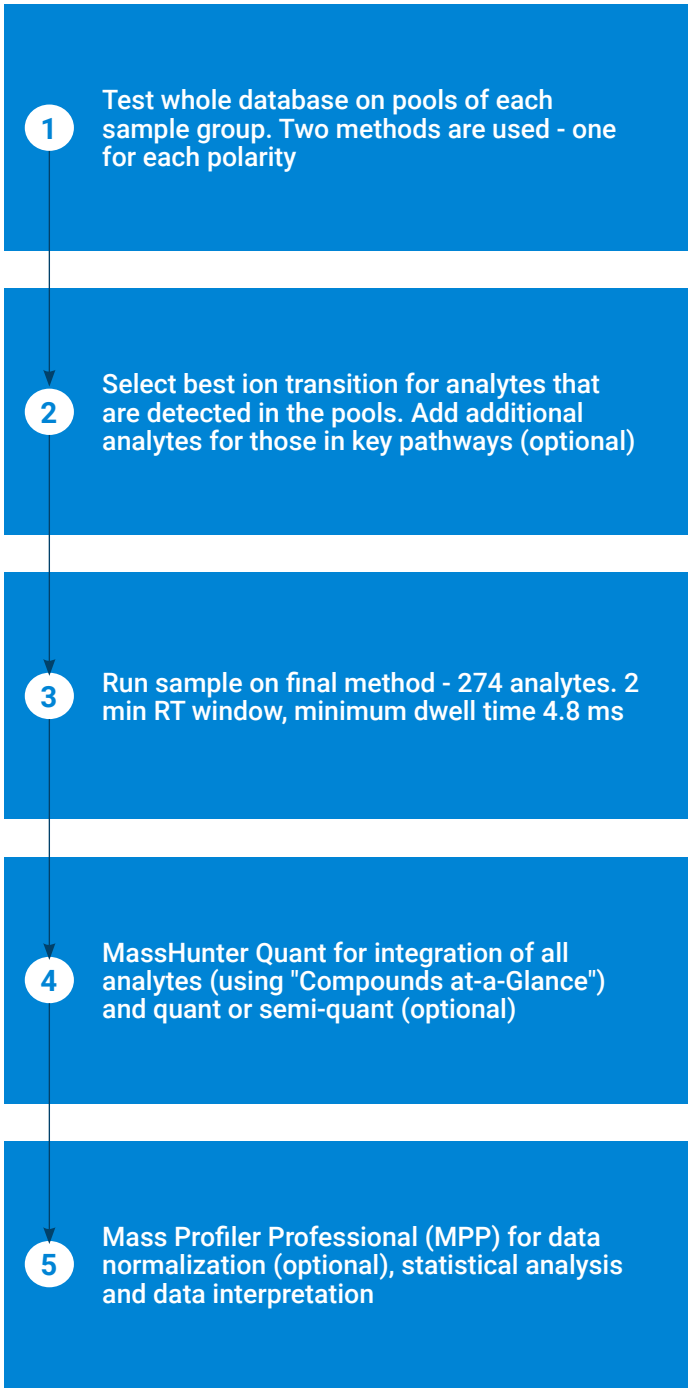

Figure 5: Workflow used to profile specific analytes present in the sample.

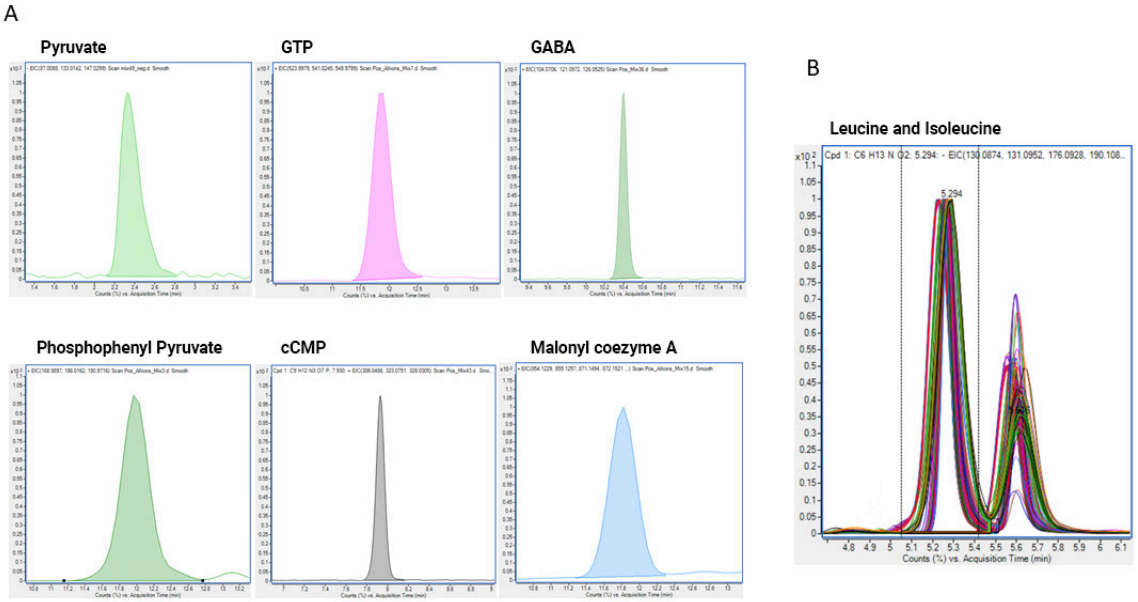

Figure 6: A. Examples of chromatograms obtained using the HILIC-Z column and standardized column and buffer preparation workflow. **B.** Overlapping of 160 chromatograms (obtained from 160 different plasma extracts) showing the reproducible separation of the isobaric analytes leucine (left) and isoleucine (right).

coverage by detecting metabolites in both ion modes. .

Reproducible retention times

One existing perception is that HILIC is not reproducible and requires advanced technical skills to be performed successfully. However, the robust workflow and standardized protocols described here ensured reproducibility across multiple end users. Figure 7 shows retention times (RT) of different analytes using different column lots, on different days, and with different batches of solvent prepared by different users. Out of over 400 injections tested, none of the metabolites had a retention time error outside of the suggested dMRM window. These results demonstrated that a HILIC dMRM method for targeted metabolomics can be

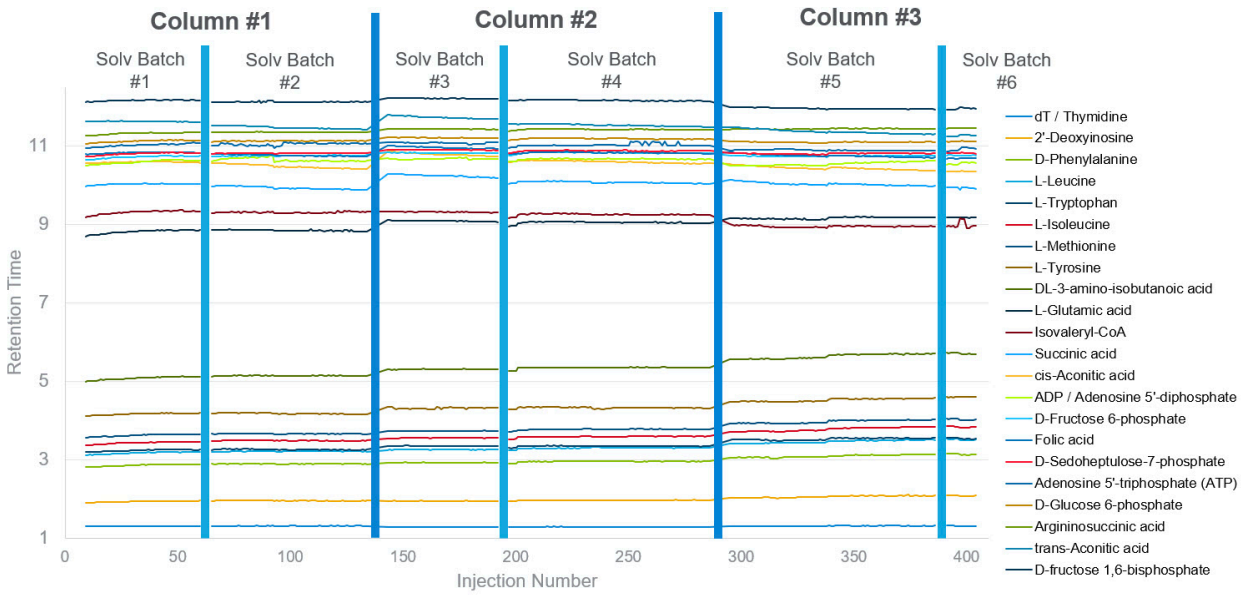

Figure 7: Retention times of different analytes using three lots of columns and six batches of solvents prepared by two different users.[8]

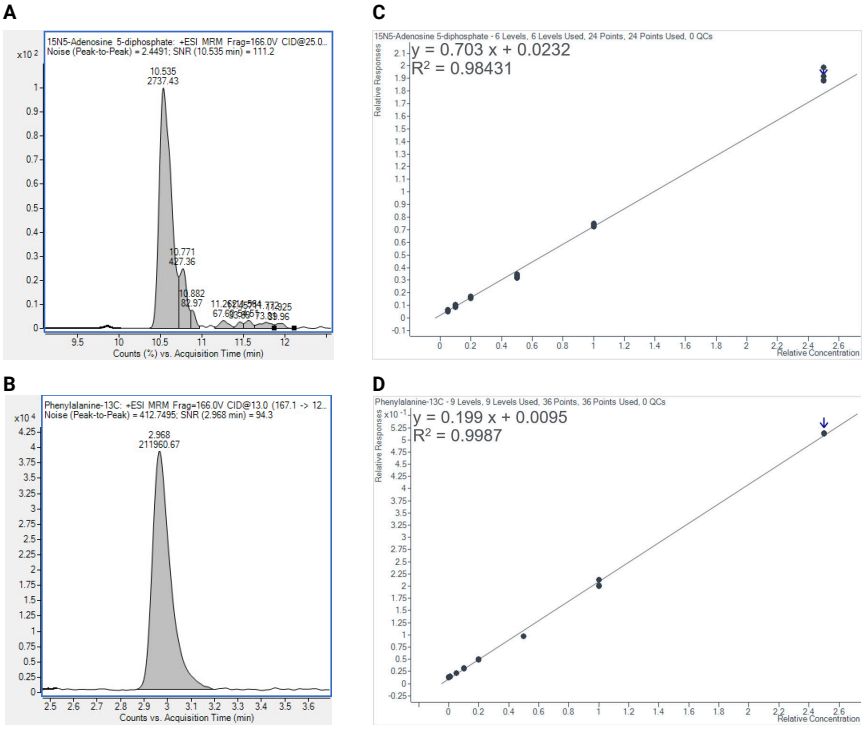

Figure 8: Absolute quantification of heavy labeled ADP and Phe from plasma extract using endogenous ADP and Phe for normalization to show the sensitivity of the analyte classes is 20 amol and 1.2 amol, respectively.

routinely implemented by many different labs, allowing users to tackle biological questions day in and day out.

High analytical sensitivity

To help maximize biological insights, the dMRM database was focused on critical metabolites, such as compounds in the TCA and glycolytic pathways, and critical coenzymes that give insights into key cellular energetic processes. Additionally, a focus on biological building blocks such as amino acids and nucleotides can help to understand the broader metabolic status of the organism. Quantification of the metabolites can be achieved using isotopically-labeled internal standards for normalization. Figure 8 shows the sensitivity of two analytes: Adenosine diphosphate (ADP) and Phenylalanine (Phe) in a plasma extract. In this experiment, different concentrations of $^{15}N_5$-Adenosine-5′-diphosphate ($^{15}N_5$-ADP) and ^{13}C-Phenylalanine (^{13}C-Phe) were spiked into pooled bovine plasma extracts. Each sample was injected four times using a method with a 5 ms dwell time. Using this method, it was possible to detect 20 amol of $^{15}N_5$-ADP with 10% RSD (Figure 8A). Amino acids had better detection limits, as it was possible to detect 1.2 amol of ^{13}C-Phe with 1% RSD (Figure 8B). In both cases, the calibration curve showed a strong linear regression (Figure 8C, R^2 = 0.98 and Figure 8D, R^2 = 0.99).

These results demonstrate that the Agilent 6495 LC/TQ instrument allows for reproducible and sensitive measurements at low dwell times. Thus, this is a robust and easy-to-use method allowing researchers to detect and quantify low amounts of analytes in a complex matrix.

Simple and fast data analysis

The MassHunter Quantitative Analysis 10 software was used to integrate all the transitions in the dataset. The software tool “Compounds-at-a-Glance” facilitates this task by displaying compounds and samples in a grid format. Those results may also be linked to the Mass Profiler Professional (MPP) software providing statistically driven biological insights. Using a wizard-based interface, MPP moves the data through the program to apply normalization, baselining, variance testing, and statistical tests. Figure 9 shows a PCA plot with clusters of six replicate injections of five unique cell matrix samples and the corresponding heat map. MPP can also provide several other plots to interrogate the trends in a sample, such as box-and-whisker plots, volcano plots, hierarchical clustering, and violin plots. Thus, the software and metabolomic database included in this workflow allow for fast and convenient data analysis, providing new biological insights more quickly.

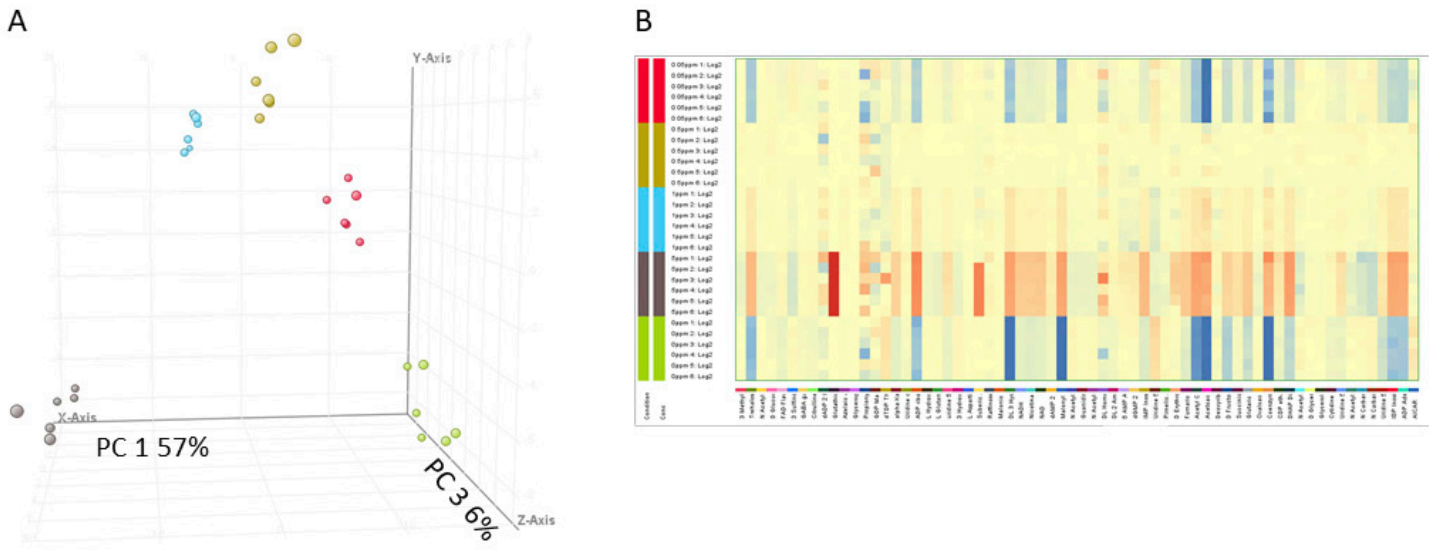

Figure 9: **A.** PCA plot showing a clear clustering of five cell matrix samples, each containing a different level of metabolite spike-ins. Green = no spike, red = 0.05 ppm spike-in, yellow = 0.5 ppm spike-in, blue = 1 ppm spike-in, and black = 5 ppm spike-in. **B.** Heat map showing the analytes that are varying in the sample, which correspond to those spiked into the cell sample.

Conclusion

Agilent solutions offer a comprehensive targeted metabolomics workflow enabling researchers to jump start their metabolomics studies. The Agilent Bravo Metabolomics Sample Prep Platform coupled with the Agilent Captiva EMR-Lipid plates allowed for efficient extraction of polar metabolites from plasma and cell samples. The extract was clean enough to be injected hundreds of times on a HILIC-Z column without chromatography degradation. A comprehensive dMRM method was built using a custom database of ion transitions and retention times for over 500 analytes. Separation using the Agilent 1290 Infinity II Bio LC system coupled with a HILIC-Z column resulted in reproducible peaks and retention times for various metabolite compound classes. The speed and analytical sensitivity of the Agilent 6495 LC/TQ instrument allowed for the detection of small amounts of hundreds of analytes at low dwell times (<1 ms). This workflow method can be customized to give customers flexibility to approach different research questions.

Learn more about Agilent 6495 Triple Quadrupole LC/MS capabilities and other targeted metabolomics solutions.

References

1. Zamboni, N.; Saghatelian, A.; Patti, G. J. Defining the Metabolome: Size, Flux, and Regulation. *Mol. Cell.* **2015**, *58(4)*, *699-706*.
2. Roberts, L. D.; Souza, A. L.; Gerszten, R. E.; Clish, C. B. Targeted Metabolomics. *Curr. Protoc. Mol. Biol.* **2012**, *30*.
3. Van de Bittner, G.; Sartain, M.; Chang, D.; Apffel, A.; Bernick, K.; Gomez, M. An Automated Dual Metabolite + Lipid Sample Preparation Workflow for Mammalian Cell Samples. *Agilent Technologies technical overview,* publication number 5994-5065EN, **2022**.
4. Spivia, W. R.; Raedschelders, K.; Gomez, M.; Van Eyk, J. E. Automated Metabolite Extraction for Plasma Using the Agilent Bravo Platform. Agilent Technologies technical overview, publication number 5994-0685EN, **2019**
5. Sartain, M.; Gomez, M.; Van de Bittner, G.; Shu, H. Enabling Automated, Low-Volume Plasma Metabolite Extraction with the Agilent Bravo Platform. *Agilent Technologies application note*, publication number 5994-2156EN, **2022**.
6. Stone, P.; Glauner, T.; Kuhlmann, F.; Schlabach, T.; Miller, K. New Dynamic MRM Mode Improves Data Quality and Triple quad Quantification in Complex Analyses. *Agilent Technologies technical overview,* publication number 5990-3595EN, **2009**.
7. Banu Mohsin, S.; Batoon, P. Absolute Quantitation of Fragile Metabolites by Isotope Dilution Mass Spectrometry on the Agilent 6495 Triple Quadrupole LC/MS. *Agilent Technologies application note*, publication number 5994-4439EN, **2022**.
8. Yannell, K. E.; Cuthbertson, D.; Simmermaker, C.; Van de Bittner, G.; Parry, E. Improvements to HILIC Robustness – a Targeted HILIC Metabolomics Method for Routine analysis. *Agilent Technologies poster,* **2021**.

www.agilent.com

RA44882.3992013889

For Research Use Only. Not for use in diagnostic procedures.

Printed in the USA, January 1, 2023
5994-5628EN

Agilent
Trusted Answers