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Quantitative Analysis of Polar Metabolites with Comprehensive Plasma and Cell Targeted Metabolomics Workflow

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Introduction

Incorporating Quantitative Analysis in a HILIC Polar Metabolomics Workflow with the New 4th Generation 6495 LC/TQ.

Targeted metabolomics methods provide a sensitive and precise measurement of metabolites with a large dynamic range. Previously described is a HILIC polar metabolite workflow using Bravo Sample Prep Platform with cells or plasma, a 1290 Infinity II Bio LC for improved performance of metal sensitive analytes, and a 6495 LC/TQ mass spectrometer with a database of ~500 polar metabolites and retention times (Figure 1).[1] The speed of the 6495 LC/TQ allows for hundreds of analytes in both positive and negative ion mode to be analyzed with precision in the same injection. This workflow and database can be deployed in several ways, from metabolite pathway discovery (profiling), to semi quantitative analysis of hundreds of analytes in a sample, or for absolute quantitation using isotopically labeled internal standards.

Figure 1: Instruments used for metabolomics workflow - Bravo Sample Prep Platform for reproducible sample prep (left),[2,3] 1290 Infinity II Bio LC (center) has improved peak shape and detection limits for metal-sensitive metabolites, and New 4th Generation 6495 LC/TQ (right) uses an ion funnel for low detection limits and low RSDs even when using 0.5 ms dwell times.

Described here is an update to the HILIC polar metabolite workflow showcasing the new 4th generation 6495 LC/TQ which has improved ion optics for increased sensitivity. The workflow update also incorporates the use of commercially available isotopically labeled internal standards for absolute quantitation of certain analytes. New 13C transitions were added to the database and quantitative performance was evaluated and described here. These transitions provide easy adoption of semi or absolute quantitative studies for researchers wanting even more precision and accuracy in their studies. Furthermore, analyte quantitation was completed on cell and plasma sample extracts processed using the Bravo Sample Prep Platform, showcasing a comprehensive quantitative workflow of complex samples.[2,3]

Experimental

Heavy Labeled Internal Standards can be Optimized from Cambridge Isotope Labs 13C Yeast Extract.

Individual analyte standards were acquired from Sigma Aldrich and prepared at high concentration in water. Cambridge Isotope Labs (CIL) yeast metabolite U-13C extract was procured and prepared in a total of 2 mL water and stored at -80°C. The neat standards were mixed into an equal-molar solution and a series of dilutions prepared. Those were further diluted to create a final calibration range of 0.1 – 100,000 nM in 70% ACN, 20% water, and 10% methanol with a 1:20 addition of the CIL 13C extract. Metabolite extract from cells (1M K562) and plasma (20 µL, mouse, BioIVT) were prepared using a Captiva EMR-Lipid SPE plate and optimized protocols for each using the Bravo Sample Prep Platform.[2,3] Each dried extract was prepared in 100 µL of ACN:H2O:MeOH (7:2:1) with a 5 µL addition of the CIL extract.

Each prepared calibrator and extract was analyzed with the 1290 Infinity II Bio LC, fit with a HILIC-Z column (2.1 x 150 mm) and coupled to the 4th generation 6495 LC/TQ. This platform supports the HILIC polar metabolite workflow, which is described elsewhere,[1] and built for retention time stability, transferability, and comprehensive analysis of metabolic pathways. Additional method details for the use of the HILIC-Z column can be found in the references.[4] New ion funnel parameters and dwell time stability of the system were explored using complex matrix and fragile analytes in this study.

Table 1. LC conditions which have proven RT reproducibility (RSD <5%) over an 11-day experiment and can be transferred to other systems.[1,4] **0.5 mL/min flow rate used to re-equilibrate the column faster.

	LC Conditions	
Column	Agilent Poroshell 120 HILIC-Z, 2.1 x 150 mm 2.7 µ, PN 683775-924	
Column temperature	15 ºC	
Injection volume	1 µL	
Autosampler temp	5 ºC	
Needle wash	Multiwash, 3 sec each: IPA, Water, and ACN	
Mobile phase*	A = 20 mM ammonium acetate, pH 9.3 + 5 µM medronic acid water B = pure ACN	
Flow rate	0.400 mL/min	
Gradient program	Time 0.00 1.00 8.00 12.00 15.00 18.00 19.00** 23	%B 90 90 78 60 10 10 90 90
Total Run Time	24 min	

MassHunter Acquisition 12 Optimizer has New Features that Make Optimizing Ion Transitions and Building Methods Easier.

MassHunter 12 has enhanced method and MRM transition Optimizer software. With a new algorithm and new features, it is now easier to optimize a method from known parameters. For some 13C metabolites, we used *a priori* knowledge from a Q-TOF study and literature for the matching 13C precursor and product ion for the metabolites. The software was efficient at only optimizing the CE for that transition (Figure 2, 'Optimize from defined range'). For another set of analytes, we had no knowledge of the product ions, so settings found in Figure 2 were used ('Find product ions from defined range'). In total, there were over 20 transitions optimized from the 13C complex mixture, using only 20 µL of the material.

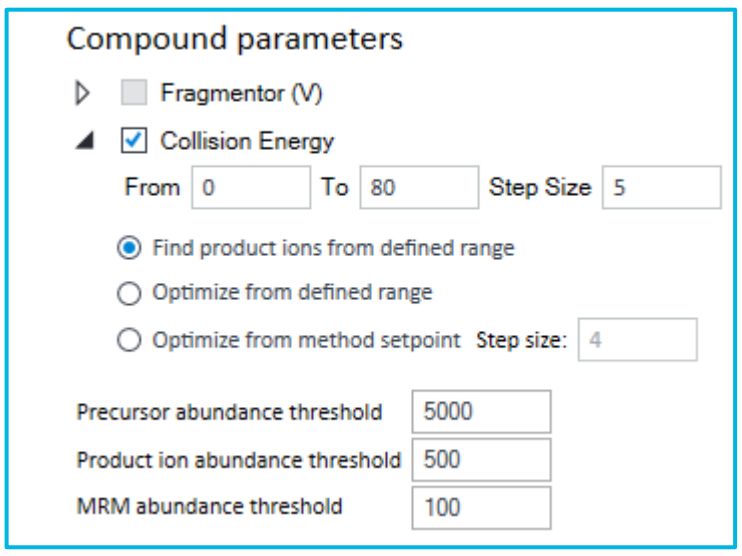

Figure 2: Screenshot from MassHunter 12 Optimization Settings showing the flexibility of optimizing transitions from different amounts of information.

A variety of analytes exist in the 12C database to cover core metabolism signaling pathways, including glycolysis, TCA, amino acid, purine, and pyrimidine pathways (500+ and counting). New 13C-labeled transitions were optimized and added for normalization and/or quantitation purposes. The calibration curve was injected 6 times each, with an additional 6 injections of the cell and plasma extracts. A dwell time of 5 ms was used for this study to assess performance at low dwell time with transitions in both ion modes (fast polarity switching). Even with this fast method, the quantitative figures of merit were excellent for the tested analytes. The lower limit of detection (LOD), limit of quantitation (LOQ or lowest calibrator), and linearity are reported in Table 2. The analytes tested with matching 13C internal standards showed very low RSD (<10%) across the calibration curve with excellent linearity across 6 orders of dynamic range - a subset of these are shown in Figure 3. Additionally, in the complex samples, the analytes were quantitated with precision using this methodology (Table 2).

Metabolites are Measured with High Sensitivity and Precision with this Workflow which Can be Used for Quantitation of Complex Matrices.

Table 2. Summary of the Quantitative Results for Analytes Measures in pos and neg ion mode, 5 ms dwell time.

Analyte	Ion Mode	LOD		Calibration Range (nM)	R^2	Plasma		Cell	
		fmol on column	RSD			nM	RSD	nM	RSD
Phenylalanine	Pos	<0.1	8%	0.1-100,000	0.9971	4,402	2%	2,828	2%
Leucine	Pos	5	12%	10-100,000	0.9999	11,110	2%	13,853	3%
N-acetyl-L-glutamic acid	Neg	0.5	4%	1-100,000	0.9999	Not Detected		71	11%
Succinic acid	Neg	5	2%	10-100,000	0.9988	2,499	1%	3,680	1%
Glycerophosphocholine	Pos	1	14%	5-100,000	0.9987	818	2%	9,325	2%
cis-Aconitic Acid	Neg	0.1	11%	0.5-100,000	0.9982	70	4%	150	2%
AMP	Pos	0.5	16%	1-100,000	0.9991	72	9%	10,038	3%
ADP	Pos	1	16%	5-100,000	0.9998	53	17%	1,053	2%
ATP	Pos	1	18%	5-100,000	0.9961	63	15%	473	3%
Sedoheptulose-7-phosphate	Neg	5	11%	10-100,000	0.9942	89	17%	1,305	7%

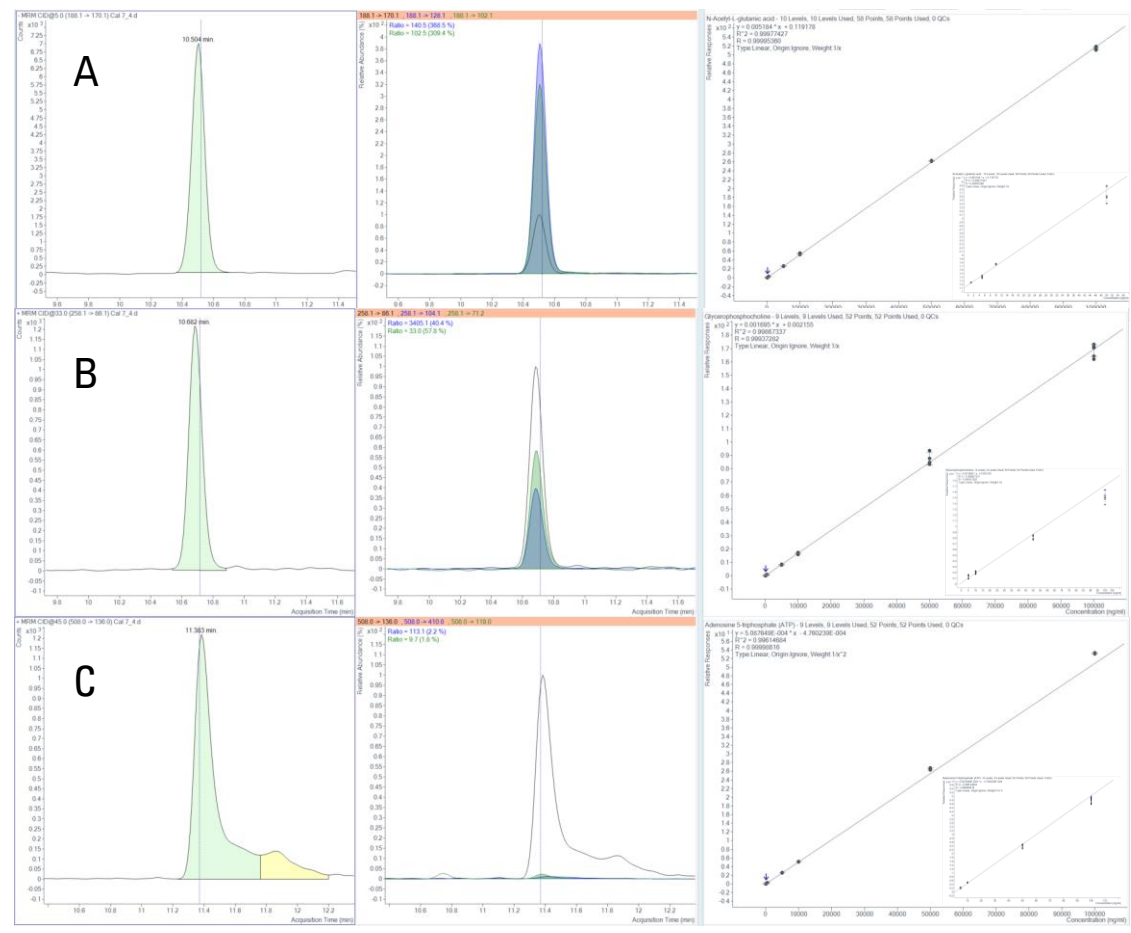

Figure 3: MassHunter Quant 12 images of the quantifier (left), qualifier(s) (center) and calibration curve (right) with the low end featured in the inset. (A) *N*-acetyl-L-glutamic acid, (B) Glycerphosphocholine, (C) ATP all show good linearity across an extended dynamic range.

Results and Discussion

New Method Optimization for Ion Funnel Parameters is Easier and Allows for Analyte Customization.

One enhancement of the 4th generation LC/TQ is the ability to simplify and customize the ion funnel parameters per analyte. Settings include Fragile, Standard, and Large Molecule. Fragile and Standard were evaluated using a cell matrix extract, running analytes using both options (n=6). The data shows that some analytes prefer the Fragile setting (Fig 4, A) while others are less specific (Fig 4, B). This gives researchers the ability to further optimize the performance for each analyte.

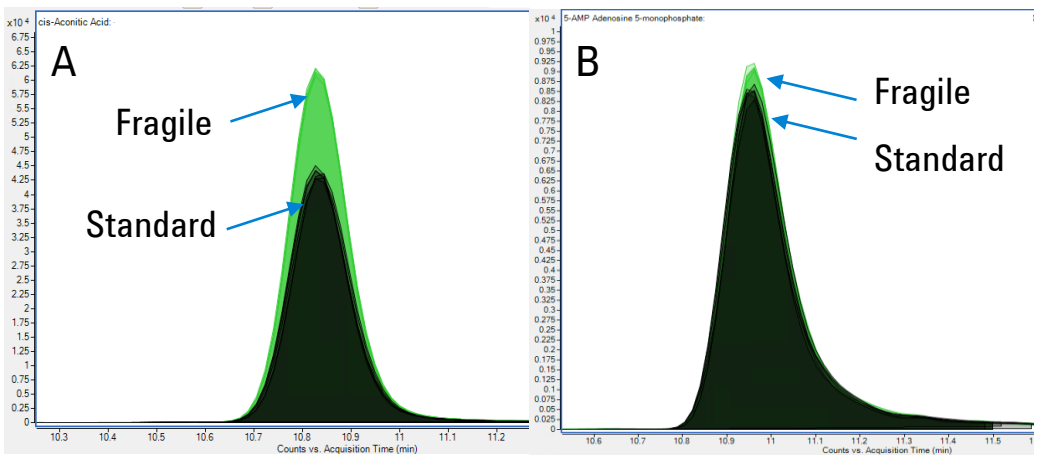

Figure 4: *cis*-Aconitic acid (A) measured with Fragile and Standard ion funnel conditions in a cell matrix (n=6) where Fragile had an 28% improvement over Standard conditions. While AMP (B) was less impacted by the settings and either condition could be used successfully.

When using the polar metabolite workflow, researchers can add as many overlapping transitions as they want up to a limit of 0.5 ms dwell time. This low dwell time, while measuring both ion polarities in the same injection, and a complex matrix pushes a LC/TQ to a limit. At this fast speed, however, a stable measurement can be measured with low RSD, even in complex matrix (Figure 5).

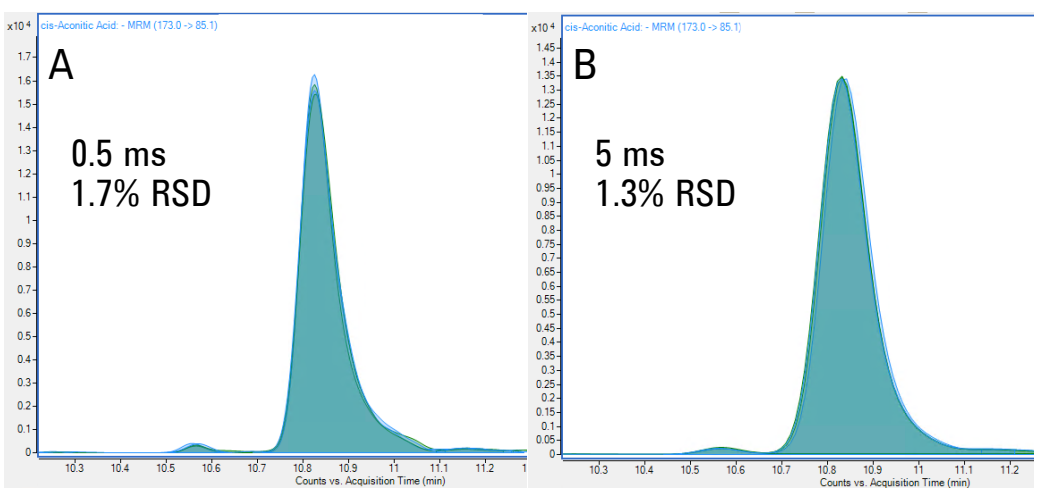

Figure 5: *cis*-Aconitic acid at 0.5 ms (A) and 5 ms (B) in a method with positive and negative ion transitions and measuring plasma extract (n=4). The RSD for both are very low suggesting stability of the measurement even when measuring hundreds of analytes in a single injection where 0.5 ms dwell time is typically used.

The New 4th Generation 6495 LC/TQ is Fast, Sensitive, Precise, and has High Dynamic Range Even when Analyzing Complex Matrix at Low Dwell Times.

The combination of this established HILIC polar metabolite workflow [1], the improved 4th Generation 6495 LC/TQ, enhanced Optimizer in MassHunter 12, and expansion of the HILIC database to include 13C transitions makes adopting metabolomics methods in your lab from pathway discovery to absolute quant much simpler to implement. This hardware, software, methods, and database are capable to be transferred to your lab to produce quality biological insights.

Conclusions

The HILIC Polar Metabolite Workflow is Enhanced with the New 4th Generation 6495 LC/TQ and Robust Quantitation is Enabled with New 13C Metabolite Transitions.

- End-to-End HILIC polar metabolite workflow can jump start your metabolomics research with methods for sample prep, HILIC chromatography, and a database with 500+ metabolites.
- New transitions added for isotopically labeled metabolites which enables easier quantitation experiments using CIL internal standard products.
- 6495 LC/TQ is fast, sensitive, precise and can measure 6 orders of dynamic range.
- Femtomole levels of metabolites are detectable at low RSD with this method and LC/TQ.
- Multiplexing a method with hundreds of co-eluting analytes is possible with precise measurements of complex samples while using a dwell time of 0.5 ms.

References

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