A Targeted LC-MS/MS Method and Acquisition Database Optimized for Central Carbon Pathway Metabolites

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Introduction

A major challenge in metabolomics is achieving reproducible, robust chromatographic resolution with a single analytical LC/MS method for endogenous cellular metabolites due to their diversity of physiochemical properties. A more manageable chromatographic solution is to develop pathway-targeted methods. We developed a highly reproducible and robust ion-pair based reverse phase (IP-RP) chromatographic method enabling simultaneous analysis of >200 endogenous cellular metabolites, along with a curated database of retention times and optimal targeted MS acquisition parameters based on chemical standards. The Agilent dMRM Database and Method enables researchers to easily implement an already-optimized method for targeted metabolomics thereby boosting laboratory productivity.

Experimental

Sample Preparation

More than 200 individual authentic chemical standards were analyzed for database curation. Cultures of Saccharomyces cerevisiae (s288c background) were grown in synthetic media containing either acetate or glucose (2% w/v) as the sole carbon source. Cultures were grown to mid-log phase (OD600=0.4), vacuum filtered onto 0.2µm nylon membranes, and immediately immersed in -20˚C ACN:MeOH:H2O (40:40:20) for quenching and extraction. Clarified metabolite extracts were dried under nitrogen and resuspended in water prior to LC-MS analysis.

UHPLC-MS/MS Method

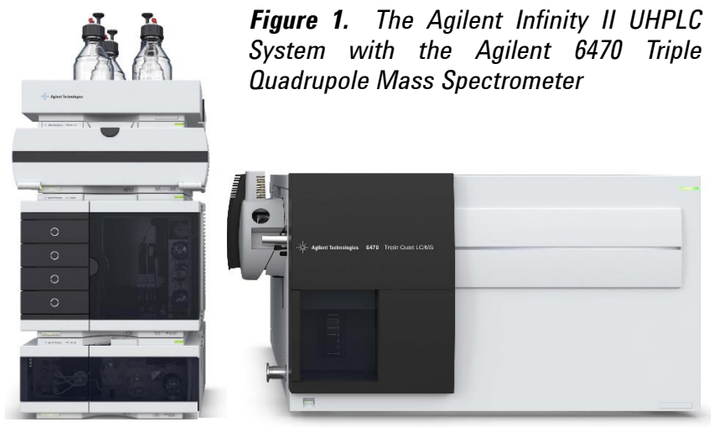

Figure 1. *The Agilent Infinity II UHPLC System with the Agilent 6470 Triple Quadrupole Mass Spectrometer*

The IP-RP method employs a UHPLC C18 column with the ion-pairing agent tributylamine (TBA) added into the LC mobile phases. The LC/MS method leverages the 1290 Infinity II UHPLC system coupled to a 6470 Triple Quadrupole LC/MS system equipped with a Jet Stream electrospray ionization source (Fig. 1), operated in dynamic multiple reaction monitoring (dMRM) mode with negative ion polarity. The method also implements a phase-matched guard column and switching valve to backflush the guard and analytical column during each run. This combination effectively clears matrix contaminants for added robustness. Major UHPLC parameters are as follows:

Agilent UHPLC 1290 Infinity II System with the 1290 Infinity II Flexible Pump (Quaternary Pump)					
Column	Agilent ZORBAX Extend C18, RRHD, 2.1x150mm 1.8 um (p/n 759700-902)				
Guard Column	Agilent ZORBAX Extend C18 Fast Guard 2.1 x 5mM, 1.8 um (p/n 821725-907)				
Column Temperature	35 °C				
Mobile Phase	A= 97:3 Water/Methanol with 10mM Tributylamine, 15mM Acetic Acid B = Isopropanol (not used) C= Methanol with 10mM Tributylamine, 15mM Acetic Acid D = Acetonitrile (backflushing solvent)				
Analytical Flow Rate	0.25 ml/min				
Gradient, Flow Rate (Analytical Portion)	Time (min)	A	B	C	D
	2.5	100%	--	0%	0%
	7.5	80%	--	20%	0%
	13.0	55%	--	45%	0%
	20.0	1%	--	99%	0%
	24.0	1%	--	99%	0%
	... backflushing and regeneration phase				
Total Run Time	40.0 min				

Results and Discussion

The Agilent dMRM Database

The Agilent Metabolomics dMRM Database provides optimized instrument operating parameters for the targeted MS acquisition of >200 metabolites. The choice of precursor and product ion ions, RTs, and RT windows were based on extensive analysis of authentic chemical standards. MassHunter (MH) Optimizer Software was used to determine optimal collision energies and fragmentor voltages for each metabolite to maximize sensitivity. The combination of these curated, optimized parameters generates results of the highest quality.

Compound transitions are directly imported into MH Acquisition software from the Database Browser to easily build tailored dMRM methods (Fig. 2). Users can also optimize new compounds of interest with MH Optimizer and results are automatically saved to the database.

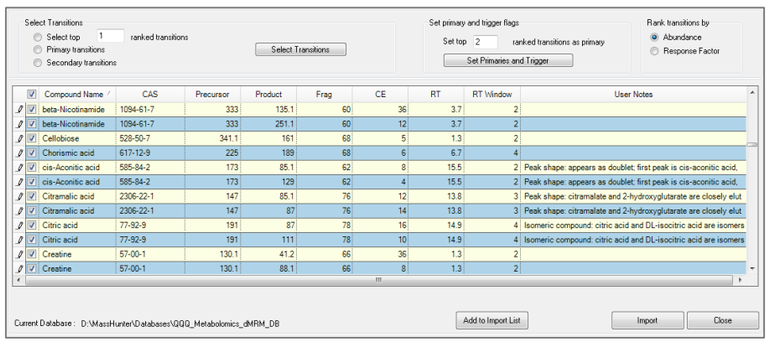

Figure 2. *Database Browser view of Metabolomics dMRM Database*

The Agilent dMRM Method

An ion-pair based reverse phase (IP-RP) chromatographic method was developed that leverages the proven robustness of Agilent C18 UHPLC columns, with added functionalization of the stationary phase with the ion-pairing agent tributylamine to facilitate retention of polar metabolites from multiple functional classes (Fig 3.). The presence of the ion-pairing ternary amine TBA in the chromatography was found to facilitate retention of acidic metabolites, while non-acidic metabolites still maintained conventional reverse-phase selectivity.

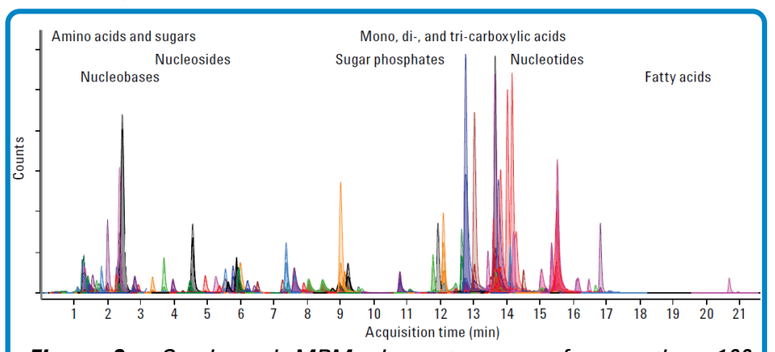

Figure 3. *Overlapped MRM chromatograms of more than 100 metabolite standards at 5 ng on-column.*

The IP-RP method is fully detailed and designed to be easily replicated across laboratories with little observed variation from the DB compound RTs, enabling narrow dMRM RT windows and providing added confidence in assigning MRM signals to the correct metabolite in a complex matrix (Fig. 4).

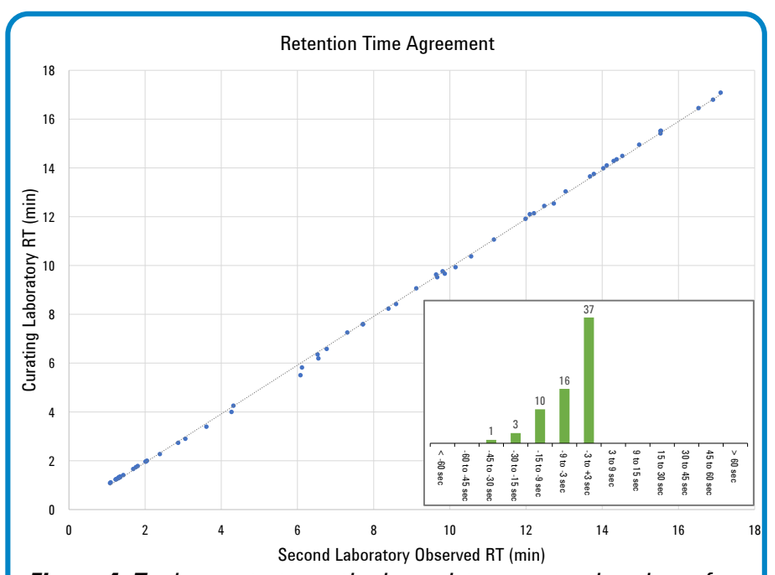

Figure 4. *To demonstrate method consistency retention times from the curating laboratory were verified at a second laboratory with a subset of standards (n=67). Excellent agreement was observed with an average Δ RT <0.1 minute. The insets shows a histogram of the compound RT deviations observed between the two laboratories.*

Results and Discussion

Data Review

With large multi-compound sample batches typically acquired with the Metabolomics dMRM method, MH Quantitative Analysis software provides easy and quick method creation for data review and peak integration. The batch type format offers helpful features such as compound-at-a-glance data review (Fig. 5). Reports can be automatically generated within the software and serve as input for the next phase of statistical analysis.

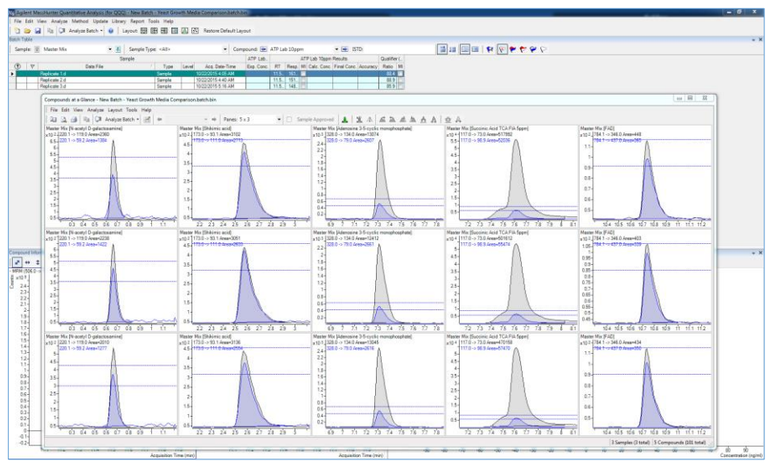

Figure 5. *Compounds-at-a-glance view within Quantitative Analysis software, showing 5 compounds (columns) across 3 samples (rows).*

Differential Analysis and Pathway Mapping

To enable multivariate relative comparisons Agilent's Mass Profiler Professional software (MPP) provides the ability to:

- Easily import, compare, and visualize LC/MS data
- Find meaningful relationships in complex experiments
- Identify affected biological pathways with the Pathway Architect module

To demonstrate the workflow, metabolite extracts from yeast cells cultured with acetate or glucose as the sole carbon source were analyzed with the full dMRM method. After processing the datasets with MH Quant and MPP, pathway analysis indicated several affected pathways including the TCA cycle (Fig. 6). Notably, pyruvate was found to be reduced in the acetate-grown cells, likely due to the lack of glucose as a substrate for glycolysis.

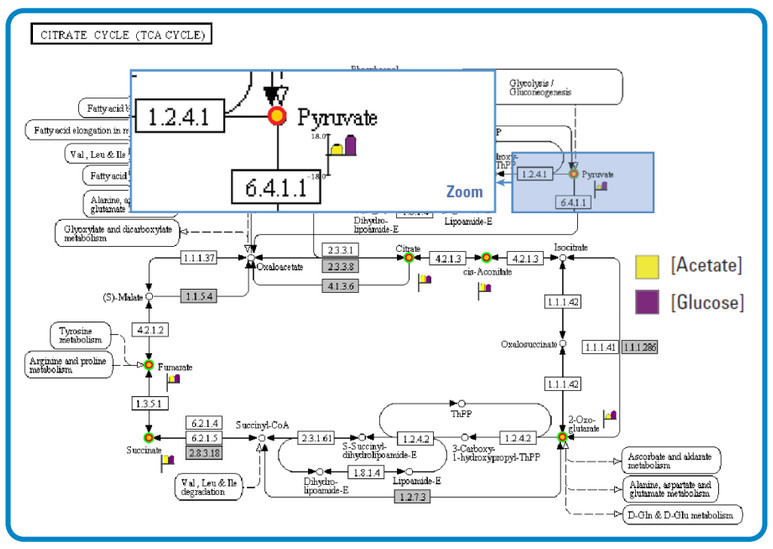

Figure 6. *Pathway Architect view of TCA Cycle (KEGG). Matching compounds are highlighted, and heat strips indicate normalized abundances.*

Conclusions

The combined UHPLC-MS/MS method and acquisition database provides:

- A robust IP-RP chromatography optimized for challenging ionic metabolites and suitable for analyses in complex cellular matrices
- Compound-specific optimized dMRM MS acquisition parameters
- Pre-defined compound retention times and retention time windows
- A customizable database format

For metabolomics researchers needing targeted methods with many metabolites per method, the database will allow fast startup and provide excellent results without the user needing to optimize each and every compound.