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Quantitation of TCA Cycle with Automated Sample Prep, Reproducible HILIC Chromatography and Ion Funnel Triple Quadrupole

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Introduction

Incorporating Absolute Quantitative Analysis of TCA Metabolites in a HILIC Polar Metabolomics Workflow with the 6495D LC/TQ.

The tricarboxylic acid (TCA) cycle has been known for several decades as a central metabolic pathway that performs the essential function of oxidizing nutrients to support cellular bioenergetics. More recently, it has become evident that TCA cycle behavior is dynamic, and products of the TCA cycle can be co-opted in cancer, other pathologic states, and in the regulation of immune responses.[1] In this study, we present a metabolomics targeted workflow for the absolute quantitation of TCA cycle metabolites in bovine plasma and cell samples using automated sample prep, a metal-free LC and a sensitive triple quadrupole mass spectrometer for robust and sensitive detection.

Figure 1. The HILIC metabolomics workflow includes reproducible metabolite extraction using the Bravo Metabolomics Sample Prep Platform (left), chromatographic separation with the Agilent Infinity II 1290 Bio LC (center), and detection with the new Agilent 6495D LC/TQ (right).

Targeted metabolomics methods provide sensitive and precise measurements of metabolites across a wide dynamic range. As previously presented, the Agilent metabolomics workflow uses a Bravo Sample Prep Platform for the extraction from cells or plasma, an Agilent 1290 Infinity II Bio LC for improved performance of metal sensitive analytes, and an Agilent 6495D LC/TQ featuring 4th generation iFunnel technology, paired with a database of over 500 polar metabolites with retention times (Figure 1) for sensitive and reproducible metabolomics analysis.[2] 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 heavy labeled internal standards.

Experimental

Building a Quantitative Method is Easy Using an MRM Database and MassHunter Optimizer.

The new MassHunter Acquisition 12.1, with built-in compound-by-compound MRM and source optimization tools, was used to determine optimal MRM and MS parameters. Individual standards of TCA metabolites were acquired from Sigma Aldrich. Cambridge Isotope Labs (CIL) yeast metabolite U-13C extract was procured and prepared in 2 mL water. The final calibration ranged from 1 to 100,000 nM in 7:2:1 ACN:H_2O:MeOH with a 1:10 addition of the CIL ^{13}C extract. Metabolite extract from cells (1M K562) and plasma (20 µL, bovine, BioIVT) were prepared using a Captiva EMR-Lipid SPE plate and optimized protocols for each using the Bravo Metabolomics Sample Prep Platform.[3,4] Each dried extract was prepared in 100 µL of ACN:H_2O:MeOH (7:2:1) with a 5 µL addition of the CIL extract. Each prepared calibrator and extract was analyzed using the HILIC-Z polar metabolite workflow, as described previously.[2,5]

Table 1. Metabolomics LC conditions.

LC Conditions	
Column	Agilent Poroshell 120 HILIC-Z, 2.1x150mm, 2.7µm PN: 683775-924
Column temp	15°C
Autosampler temp	4°C
Needle wash	Multiwash: 3s each: IPA, H_2O, ACN
Mobile phase	**A:** 20mM ammonium acetate, pH 9.3 + 5µM medronic acid **B:** Pure ACN
Flow rate	0.4 mL/min
Gradient program	0.0 – 1.0 min (90%B), 1.0 – 8.0 min (90 – 78%B), 8.0 – 12.0 min (78 – 60%B), 12.0-15.0 min (60-10%B), 15.0 – 18.0 min (10 %B), 18.0 – 19.0 min (10 - 90%B)
Total run time	24 min

Table 2. New MS parameters

6495D MS Conditions	
Sheath Gas Temperature	400 ºC
Sheath Gas Flow	12.0 L/min
Gas Temperature	275 ºC
Gas Flow	13.0 L/min
Nebulizer	40.0 psi
Capillary	3000 V (+) / 2000 V (-)
Nozzle Voltage	500 V (+) / 0 V (-)
iFunnel Mode	Fragile
Detector Gain	2

New Sensitive Method for Absolute Quantitation of TCA Metabolites Using a Reproducible HILIC-Z Metabolomics Workflow

To facilitate targeted quantitation analysis, new transitions for ^{13}C-labeled TCA metabolites were added to the HILIC metabolomics database, which features an additional 1700+ ^{12}C transitions covering 500+ metabolites. Twelve TCA metabolites and corresponding ^{13}C-labeled standards were selected for absolute quantitation analysis (Figure 2).

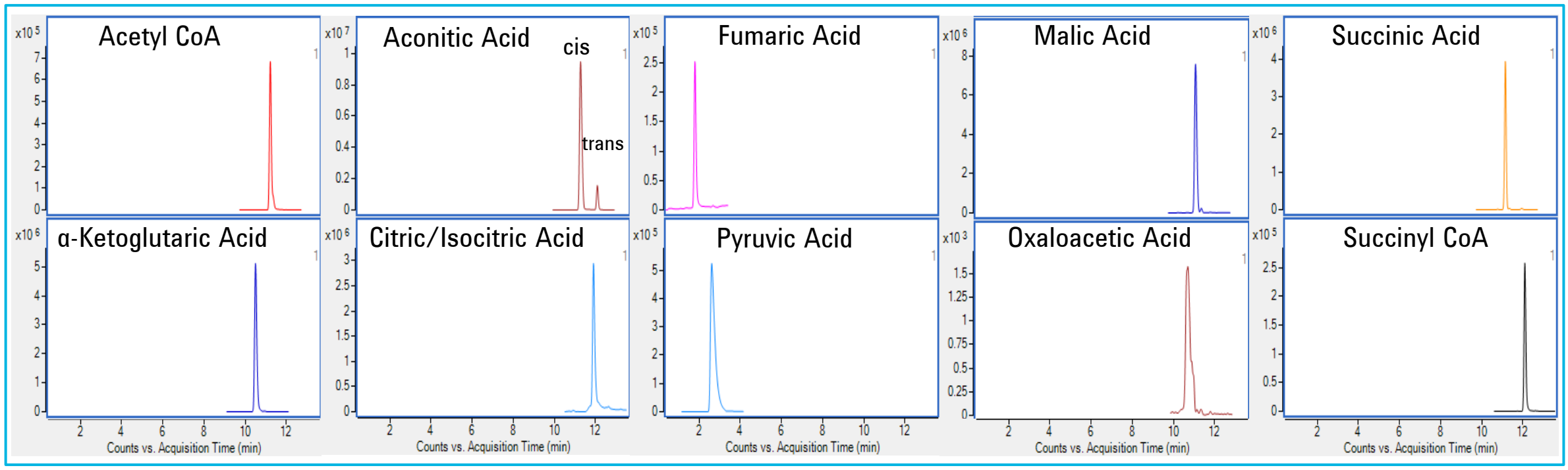

Figure 2: Chromatographic separation of TCA cycle compounds using HILIC-Z column.

One enhancement of the 4th generation ion funnel on the 6495D LC/TQ is the ability to customize the ion funnel parameters on an analyte-by-analyte basis. Fragile and Standard modes were evaluated using a mid-range calibrant, with both iFunnel settings (n=6). The majority of TCA metabolites showed improved sensitivity with the Fragile iFunnel mode (Figure 3).

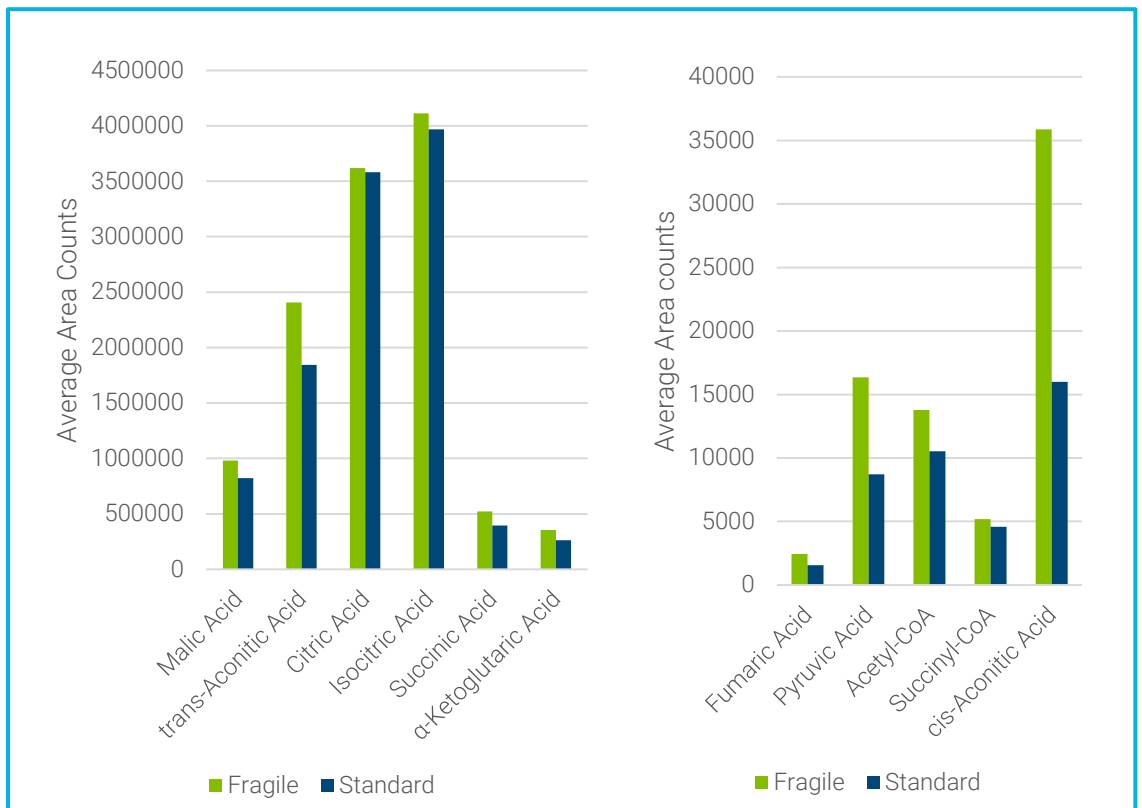

Figure 3: Fragile iFunnel mode showed improved sensitivity for all TCA metabolites, with enhanced performance ranging from 1% (citric acid) to 124% (*cis*-aconitic acid).

The calibration curve and samples were injected 6 times each. The quantitative figures of merit were excellent for the tested analytes as reported in Table 3. The analytes tested with matching ^{13}C internal standards showed very low RSD (<10%) across the calibration curve with excellent linearity, as shown in Figure 4.

Table 3. Summary of the quantitative results for analytes measured in positive and negative ion mode.

Compounds	Ion Mode	RT (min)	Cal Curve range (nM)	R^2	RSD (%)
Pyruvic Acid	Neg	2.7	1 - 100000	0.999	3.19
cis-Aconitic Acid	Neg	10.0	1 - 100000	0.999	9.43
α-Ketoglutaric Acid	Neg	10.5	5 - 10000	0.998	6.25
Oxaloacetic Acid	Neg	10.7	100 - 10000	0.995	9.67
Fumaric Acid	Neg	11.1	5 - 50000	0.999	2.64
Succinic Acid	Neg	11.1	10 - 100000	0.999	2.22
Malic Acid	Neg	11.2	10 - 50000	0.998	1.69
Iso/Citric Acid	Neg	11.9	5 – 100000	0.998	2.24
Succinyl CoA	Pos	12.1	10 - 100000	0.998	5.41
trans-Aconitic Acid	Neg	12.1	1 - 100000	0.999	5.01
Acetyl CoA	Pos	12.2	1 - 100000	0.999	16.67

Results and Discussion

Metabolites are Measured Sensitively and Precisely with Metabolomics Workflow in Complex Matrices.

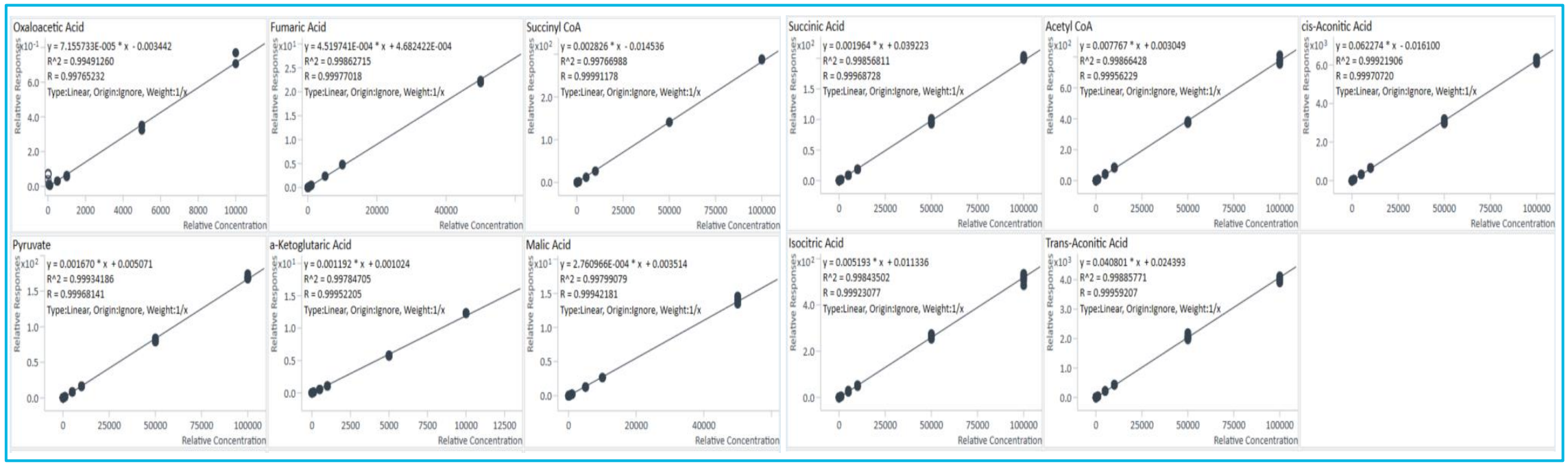

Figure 4: MassHunter Quant 12.1 analysis generates calibration curve at-a-glance. Figures allow quick processing of all calibration curves and customizable visualization to fit your compounds.

TCA analytes were detected in both bovine plasma and cell matrix metabolite extracts. Measured TCA metabolite concentrations in both matrices were all within calibration curve ranges. Table 4 summarizes all TCA metabolites extracted and detected in both matrices.

Table 4. Quantitative results for TCA metabolites measured in complex matrices.

Compounds	Bovine Plasma		Cell (K562)	
	nM	RSD (%)	nM	RSD (%)
Pyruvic Acid	815	7.40	690	3.28
cis-Aconitic Acid	40	6.79	253	1.65
α-Ketoglutaric Acid	2977	1.93	3737	2.27
Oxaloacetic Acid	n.d.		1392	8.24
Fumaric Acid	376	4.35	6741	1.46
Succinic Acid	1902	3.23	3039	1.14
Malic Acid	465	3.92	9556	1.87
Iso/Citric Acid	719	3.75	2136	2.36
Succinyl CoA	n.d.		11	9.22
trans-Aconitic Acid	11	8.39	60	8.33
Acetyl CoA	1	13.13	161	4.14

Conclusions

New, Optimized Transitions and Enhanced Sensitivity for a Comprehensive Metabolomics Workflow – from Screening to Absolute Quantitation.

- Newly optimized transitions for ^{13}C-labeled TCA metabolites facilitate absolute quantitation in complex matrices.
- Fragile iFunnel settings improved sensitivity for the TCA metabolites.
- The new 6495D LC/TQ is fast, sensitive, precise, and can measure 6 orders of dynamic range.
- 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, for highly sensitive profiling and/or quantitation.

References

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