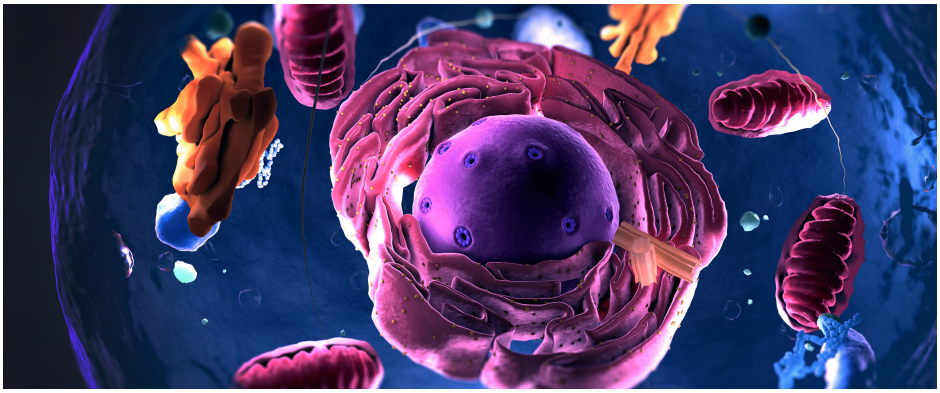
Application Note

Clinical Research

Quantitation of TCA Cycle Metabolites with LC/TQ and Standardized HILIC Chromatography

Analyzing TCA metabolites in bovine plasma and cell samples using the Agilent 6495D triple quadrupole LC/MS and the Agilent 1290 Infinity II bio LC system



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Abstract

This application note describes a targeted metabolomics workflow for the absolute quantitation of tricarboxylic acid (TCA) cycle metabolites in bovine plasma and cell samples. An Agilent 1290 Infinity II bio LC system and an Agilent 6495D triple quadrupole LC/MS system were used as part of a standardized HILIC metabolomics method for sensitive detection of TCA metabolites.

Introduction

The 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, an iron-free 1290 Infinity II bio LC, and a 6495D LC/TQ for robust and sensitive detection.

Targeted metabolomics methods provide sensitive and precise measurements of metabolites across a wide dynamic range. Agilent's end-to-end targeted metabolomics workflow uses the Agilent Bravo Metabolomics Sample Prep Platform for the extraction from cells or plasma, a 1290 Infinity II bio LC for improved performance of metal-sensitive analytes, and a 6495D LC/TQ featuring fourth-generation Agilent iFunnel technology, paired with a database of over 500 polar metabolites with retention times 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 absolute quantitation using heavy-labeled internal standards. Quantitation of TCA metabolites is achieved with the standardized HILIC metabolomics methodology.

Experimental

Sample preparation

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 extracts from cells (1 M K562) and plasma (20 µL, bovine, BioIVT) were prepared using an Agilent Captiva EMR–Lipid SPE plate and optimized protocols for both matrices using the Bravo Metabolomics Sample Prep Platform.[3,4] Each dried extract was reconstituted 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]

Equipment

This experiment was conducted using the following instrument configuration:

- Agilent 6495D triple quadrupole LC/MS (G6495D)
- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity II diode array detector HS (G7117C)

Although this analysis used a 1290 Infinity II bio LC configuration, comparable results can be achieved on the 1290 Infinity III bio LC system with no changes to method parameters.

Methods

Liquid chromatography:

Table 1. Agilent 1290 Infinity II bio LC parameters.

1290 Infinity II Bio LC System	
Column	Agilent Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 µm (p/n 683775-924)
Sampler Temperature	4 °C
Mobile Phase A	20 mM ammonium acetate, pH 9.3 + 5 µM InfinityLab deactivator in water
Mobile Phase B	Acetonitrile
Flow Rate	0.4 mL/min
Injection Volume	4 µL
Column Temperature	15 °C
Gradient Program	Time (min) %B 0 90 1.0 90 8.0 78 12.0 60 15.0 10 18.0 10 19.0 90
Post-Run Time	5 min

Mass spectrometry:

Table 2. Agilent 6495D triple quadrupole LC/MS parameters.

6495D Triple Quadrupole LC/MS System	
Ion Source	Agilent Jet Stream ESI
Polarity	Positive and negative
Gas Temperature	275 °C
Drying Gas Flow	13.0 L/min
Nebulizer	40.0 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	12.0 L/min
Capillary Voltage	Positive: 3,000 V Negative: 2,000 V
Nozzle Voltage	Positive: 500 V Negative: 0 V
Detector Gain Factor	2
iFunnel Mode	Fragile

Compound information and MRM settings:

Table 3. Detailed MRM settings and compound information for TCA analytes.

Compound Name	Precursor *m/z*	Product *m/z*	RT (min)	CAV (V)	CE (V)	iFunnel Mode	Polarity
13C_Acetyl CoA	833.2	316.3	11.23	2	25	Fragile	Positive
13C_a-Ketoglutaric Acid	150	105	10.6	2	3	Fragile	Negative
13C_cis-Aconitic Acid	179	134	11.42	2	3	Fragile	Negative
13C_Citric Acid	197	116	12.01	2	11	Fragile	Negative
13C_Fumaric Acid	119.1	74.1	11.2	2	9	Fragile	Negative
13C_Isocitric Acid	197	161.1	11.62	2	12	Fragile	Negative
13C_Malic Acid	137	119	11.65	5	8	Fragile	Negative
13C_Oxaloacetic Acid	135	91	10.8	2	12	Fragile	Negative
13C_Pyruvate	90.1	45.2	2.69	2	5	Fragile	Negative
13C_Succinic Acid	121	76	11.22	2	11	Fragile	Negative
13C_trans-Aconitic Acid	179	134	12.2	2	3	Fragile	Negative
Acetyl CoA	810.1	303.2	11.23	5	55	Fragile	Positive
Acetyl CoA	810.1	159	11.23	5	60	Fragile	Positive
a-Ketoglutaric Acid	145	101	10.6	5	5	Fragile	Negative
a-Ketoglutaric Acid	145	57.1	10.6	5	10	Fragile	Negative
cis-Aconitic Acid	173	129	11.42	5	5	Fragile	Negative
cis-Aconitic Acid	173	85.1	11.42	5	10	Fragile	Negative
Citric Acid	191	172.9	12.01	5	5	Fragile	Negative
Citric Acid	191	111	12.01	5	10	Fragile	Negative
Fumaric Acid	115	71	11.2	5	5	Fragile	Negative
Fumaric Acid	115	27.2	11.2	5	10	Fragile	Negative
Isocitric Acid	191.1	111	11.99	5	11	Fragile	Negative
Isocitric Acid	191.1	85.1	11.99	5	13	Fragile	Negative
Malic Acid	133	115	11.25	5	10	Fragile	Negative
Malic Acid	133	71.1	11.25	5	15	Fragile	Negative
Oxaloacetic Acid	130.9	87.1	10.52	5	10	Fragile	Negative
Pyruvate	87	43.1	2.69	5	5	Fragile	Negative
Pyruvate	87	41.2	2.69	5	25	Fragile	Negative
Succinic Acid	117	98.9	11.22	5	7	Fragile	Negative
Succinic Acid	117	73	11.22	5	11	Fragile	Negative
Succinyl CoA	868.1	142	12.1	5	80	Fragile	Positive
Succinyl CoA	868.1	136.1	12.1	5	65	Fragile	Positive
trans-Aconitic Acid	173	85.1	12.2	5	10	Fragile	Negative
trans-Aconitic Acid	173	41.2	12.2	5	15	Fragile	Negative

Results and discussion

To facilitate targeted quantitation analysis, new transitions for ^{13}C-labeled TCA metabolites were added to the HILIC metabolomics database, which features more than 1,800 ^{12}C transitions covering more than 500 metabolites. Twelve TCA metabolites and corresponding ^{13}C-labeled standards were selected for absolute quantitation analysis (Figure 1).

One enhancement of the fourth-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). Most TCA metabolites showed improved sensitivity with the Fragile iFunnel mode (Figure 2).

The calibration curve and samples were injected six times each. The quantitative figures of merit were excellent for the tested analytes, as reported in Table 4. The analytes tested with matching ^{13}C internal standards showed very low RSD (< 10%) across the calibration curve, with excellent linearity. Agilent MassHunter Quantitative Analysis 12.1 generates calibration curves at a glance, allowing for quick processing and customizable visualization to fit your components (Figure 3).

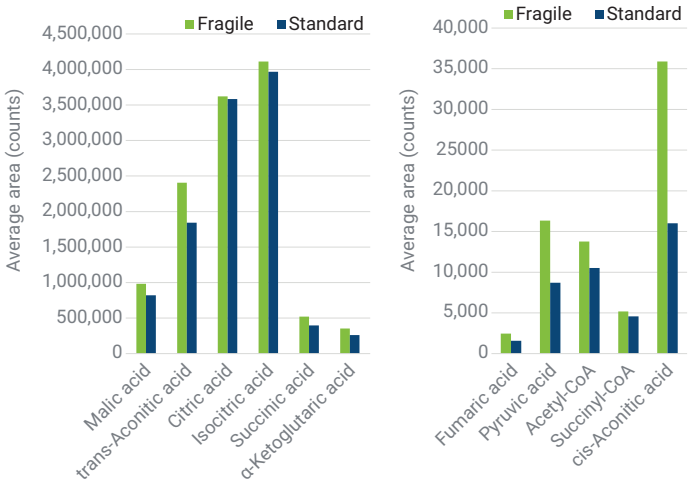

Figure 2. Comparison of Fragile and Standard Agilent iFunnel modes for TCA metabolites.

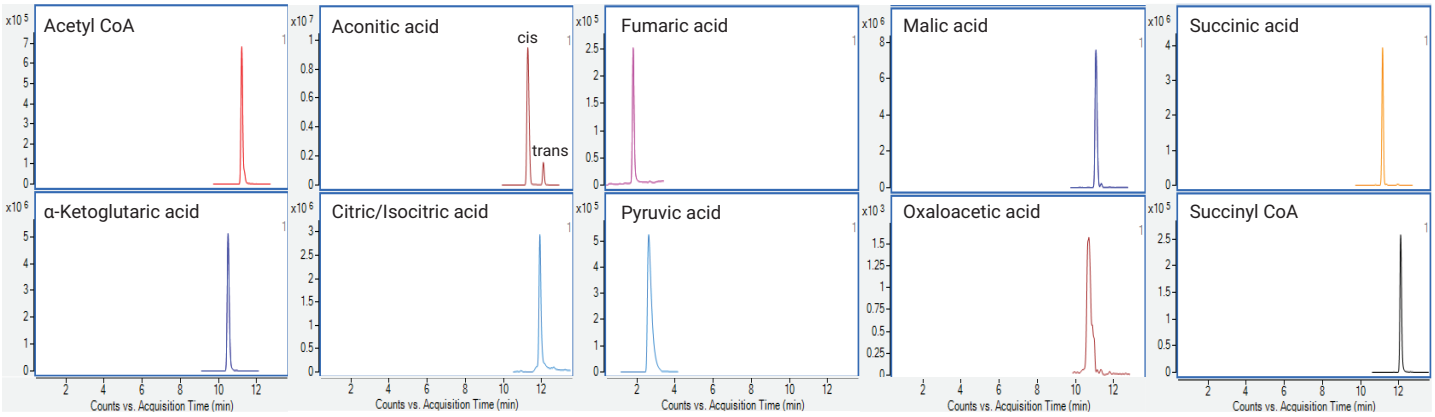

Figure 1. Chromatographic separation of TCA cycle compounds.

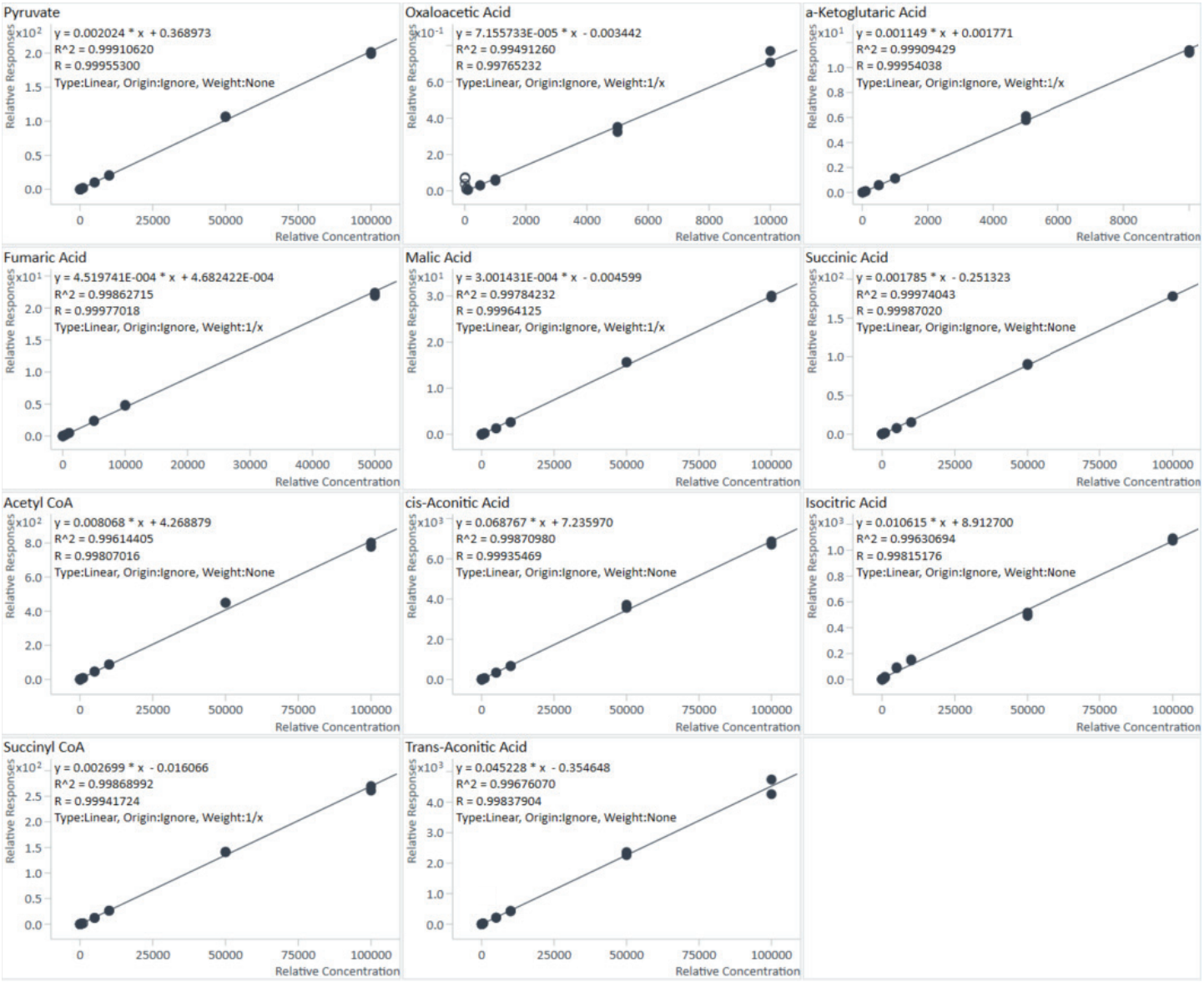

Figure 3. Calibration curves of TCA cycle analytes.

Table 4. Summary of calibration curve results for TCA analytes.

Compounds	Ion Mode	RT (min)	Calibration Curve Range (nM)	R^2	RSD (%)
Pyruvic Acid	Negative	2.7	1–100,000	0.999	3.19
cis-Aconitic Acid	Negative	10.0	1–100,000	0.999	9.43
α-Ketoglutaric Acid	Negative	10.5	5–10,000	0.998	6.25
Oxaloacetic Acid	Negative	10.7	100–10,000	0.999	3.19
Fumaric Acid	Negative	11.1	5–50,000	0.999	9.43
Succinic Acid	Negative	11.1	10–100,000	0.998	6.25
Malic Acid	Negative	11.2	10–50,000	0.995	9.67
Iso/citric Acid	Negative	11.9	5–100,000	0.999	2.64
Succinyl CoA	Positive	12.1	10–100,000	0.998	5.41
trans-Aconitic Acid	Negative	12.1	1–100,000	0.999	5.01
Acetyl CoA	Positive	12.2	1–100,000	0.999	3.19

TCA cycle 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 5 summarizes all TCA metabolites extracted and detected in both matrices.

Table 5. Quantitation results for TCA analytes in bovine plasma and K652 cell extracts. RSD = relative standard deviation; n.d. = not detected.

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	2,977	1.93	3,737	2.27
Oxaloacetic Acid	n.d.		1,392	8.24
Fumaric Acid	376	4.35	6,741	1.46
Succinic Acid	1,902	3.23	3,039	1.14
Malic Acid	465	3.92	9,556	1.87
Iso/citric Acid	719	3.75	2,136	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

Conclusion

This application note applies newly optimized transitions for ^{13}C-labeled TCA metabolites that facilitate absolute quantitation in complex matrices. The use of Fragile-mode Agilent iFunnel settings improved sensitivity for all TCA metabolites.

The new Agilent 6495D triple quadrupole LC/MS is fast, sensitive, precise, and can measure six orders of dynamic range.

This end-to-end HILIC polar metabolite workflow can jump start your metabolomics research with methods for sample preparation, HILIC chromatography, and a database with over 500 metabolites, for highly sensitive profiling and quantitation.

References

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