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Simultaneous Detection of Tricarboxylic Acid Cycle Intermediates using LC-MS/MS with a Synergi™ Fusion-RP HPLC Column

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Introduction

The tricarboxylic acid (TCA) cycle is a critical metabolic pathway present in a majority of living organisms.[1] In addition to its importance to normal life function, the TCA cycle has been the focus of additional research because of its implications in metabolomics for cancerous cells. For instance, several research groups have studied the metabolism of citrate between healthy and cancerous cells for diagnosis purposes and targeting of cancer therapies.[2,3] Because of the importance of the TCA cycle, several methodologies have been proposed for the analytical monitoring of metabolites from a range of biological matrices.

Presently, many of the published methods for the analysis of TCAs utilizes either enzymatic, Nuclear Magnetic Resonance (NMR), or Liquid Chromatography-Mass Spectroscopy (LC-MS) as the analytical technique for the detection and quantification of the cycle's intermediates.[4,5] However, there exists significant challenges to the detection and quantification of these metabolites within the aforementioned analytical techniques. For instance, enzymatic techniques often rely on indirect detection of the intermediates and therefore provides less direct information about TCAs.[6] In the case of NMR, it does allow for direct monitoring, however it lacks the same sensitivity as LC-MS/MS and is generally a less available technique.

Therefore, presented in this technical note is a reference method for the simultaneous reversed phase analysis of TCA intermediates by LC-MS/MS with a focus on tricarboxylic acids (Citric, Isocitric, Malic, Succinic, Lactic, Glutamic and Fumaric acid) and a deuterated internal standard (Citric acid-D4) in a human biological matrix.

The analytical challenges were defined as adequate reversed phase chromatographic retention, a mobile phase system compatible with mass spectroscopy (MS) detection, baseline separation of the critical isomers Citric/isocitric acid, inherently high endogenous TCA levels within biological matrices, overall analyte stability, peak shape, and TCA's pH sensitivity.

In this technical note, the goal was to develop an LC-MS/MS assay able to analyze tricarboxylic acids out of a human serum albumin matrix over a dynamic range of concentrations and demonstrate acceptable accuracy and precision in reference to GLP guidance. In addition, the reference method should be a fast and reproducible assay, easily implemented in most laboratories with standard analytical equipment.

Experiment

Analytical reference standards for Citric, Isocitric, Malic, Succinic, Lactic, and Glutamic acid, deuterated internal Citric acid standard, Dulbecco's phosphate buffered saline and human serum albumin powder (HSA, fatty acid free) were obtained through Sigma-Aldrich®. The Synergi Fusion-RP, a fully porous polar embedded C18 with trimethylsilyl (TMS) end-capping was selected after an extensive column screening was performed to determine the most most applicable selectivity. An Agilent® 1260 Infinity HPLC system was used for this investigation and a SCIEX® Triple Quad™4500 MS/MS was used for detection. The Triple Quad 4500 was equipped with an ESI source capable of in-analysis polarity switching and eQ™ electronics which can polarity switching in 50 ms and has scan speeds of 20,000 Da/s. This allowed for simultaneous detection of TCA using both negative and positive ionization mode in one run.

Sample preparation of the human serum albumin (HSA, fatty acid free) that was used for all standards and QC's preparation, consisted of taking 420 mg of HSA and dissolving it in 12 mL of Dulbecco's phosphate buffered saline solution (35 mg/mL), mixing, and then stored between 2 – 8 °C. An internal standard solution of Citric acid-D4 was prepared in 0.1 % Formic acid at a concentration of 5 µg/mL.

Preparation of standards consisted of two sets of standards at an eight-point concentration range of 20, 45, 150, 300, 500, 800, 1000, and 2000 ng/mL. Six sets of Quality Control (QC) samples were prepared at four concentrations of 60, 200, 800, and 1500 ng/mL, and extracted.

Acidified protein precipitation was used for sample extraction. First, 10 µL of spiked human serum albumin was added to a 1.8 mL microcentrifuge tube, then 10 µL of working internal standard (Citric acid-D4 at 5 µg/mL) was added and mixed with 100 µL 5 % Trichloroacetic acid) for approximately 1 minute. The mixture was then centrifuged at 18000 rpm for 10 minutes. 100 µL of the supernatant was transferred to a Verex™ autosampler vial and positioned into the autosampler.

The mobile phase consisted of a premixed ratio of Water/Methanol (95:5) with 0.2 % Formic acid added to the aqueous portion. The column heating oven was set to 45 °C, injection volume at 10 µL, and a flow rate of 0.85 mL/min was used for this example.

Mass Spectrometer ESI source parameters are referenced in **Table 1** and **Table 2** and additionally contain the mass transitions used for this analysis.

Table 1.
ESI ionization source parameters.

Source/Gas Parameters	NEGATIVE ION	POSITIVE* ION
Curtain Gas (CUR):	20	20
Collision Gas (CAD):	6	6
Temperature (TEM):	700	700
Ion Source Gas 1 (GS1):	50	50
Ion Source Gas 2 (GS2):	50	50
IonSpray Voltage (IS):	-4000	4000
Entrance Potential (EP):	-10	10
Collision Cell Exit Potential (CXP):	-10	10

*Glutamic acid was analyzed in positive ionization mode, all others were under negative ionization mode

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Table 2.
TCA mass transitions.

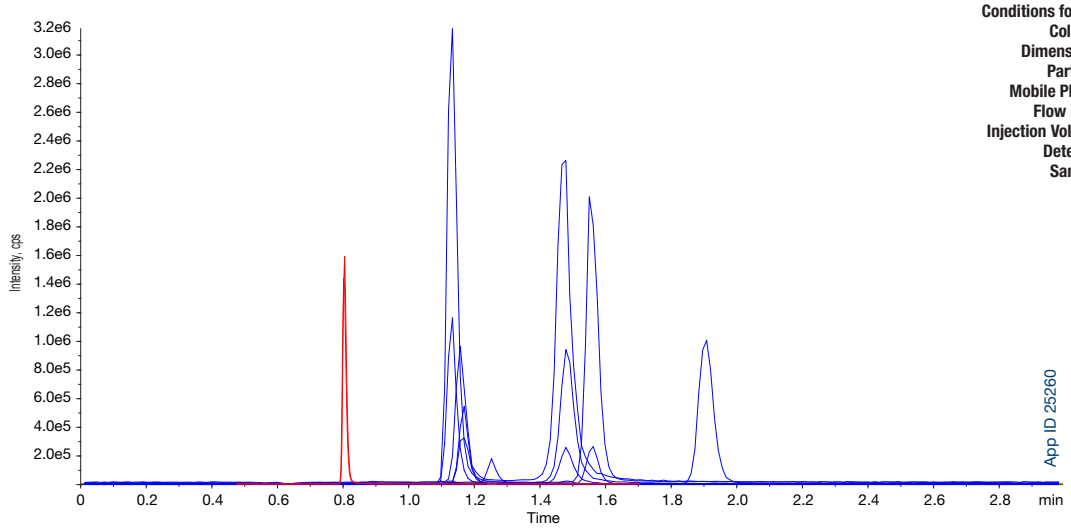
ID	Q1 Mass (Da)	Q3 Mass (Da)	Dwell (msec)	DP	CE
Citric acid 1	191	87	25	-35	-22
Citric acid 2	191	111	25	-35	-12
Isocitric acid 1	191	155	25	-35	-18
Isocitric acid 2	191	129	25	-30	-18
Malic acid 1	133	115	25	-35	-14
Malic acid 2	133	71	25	-35	-19
Succinic acid 1	117	99	50	-30	-14
Succinic acid 2	117	73	50	-30	-15
Fumaric acid 1	115	71	50	-25	-10
Fumaric acid 2	115	98	50	-25	-10
Lactic acid 1	89	43	50	-34	-20
Lactic acid 2	89	41	50	-34	-20
IS- Citric acid-D4	195	114	50	-35	-12
Glutamic acid 1+	148	102	50	40	15
Glutamic acid 2+	148	84	50	40	20

+ positive ion

Results

Figure 1 displays a neat standard solution of seven TCAs and internal standard at a concentration of 500 ng/mL and with an overlay that shows all analytes in both negative and positive mode. **Figure 2** is a representative chromatogram of the Quality Control High (QCH) standard at 1500 ng/mL in human serum albumin without Fumaric acid due to the compound stability issue in the matrix of HSA.

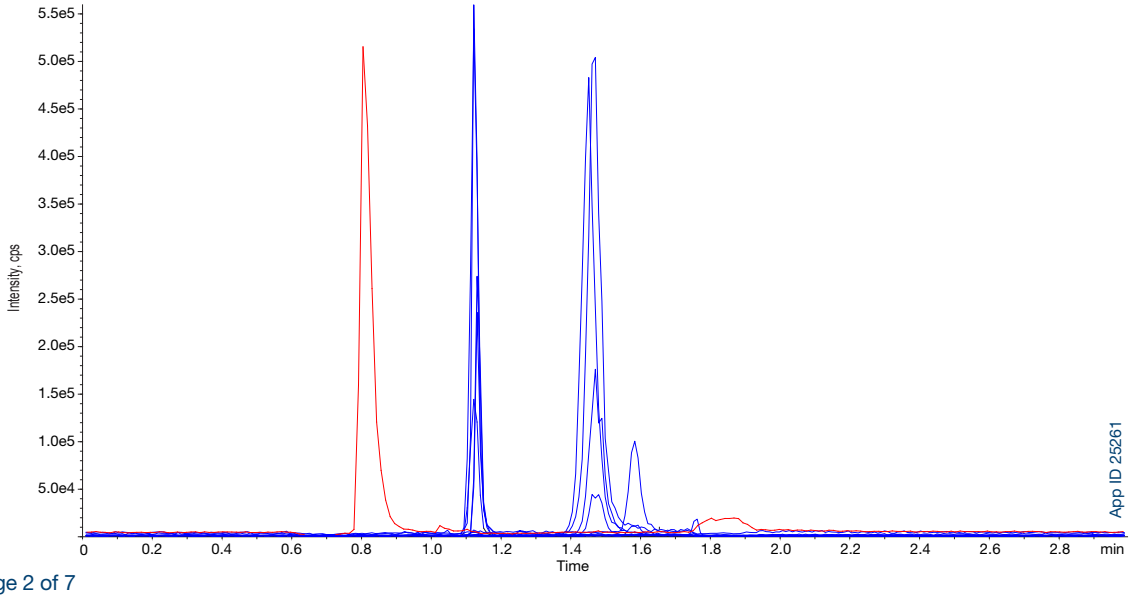
Figure 1. TCA standards (500 ng/mL).



Conditions for Figure 1 & 2

Column: Synergi™ 4 µm Fusion-RP
Dimensions: 150 x 3.0 mm
Part No.: 00F-4424-Y0
Mobile Phase: Methanol/ Water with 0.2 % Formic acid (95:5)
Flow Rate: 0.85 mL/min
Injection Volume: 10 µL
Detector: MS/MS SCIEX™ 4500™, ESI with polarity switching
Sample: 1. Glutamic acid (POS ion mode)
2. Malic acid
3. Isocitric acid
4. Lactic acid
5. Citric acid-D_4 (IS)
6. Citric acid
7. Succinic acid
8. Fumaric acid

Figure 2. QCH (1500 ng/mL) in HSA.



Figures 3 & 4 are representative chromatograms of blank human serum albumin **Figure 3** versus the lower limit of quantitation in a matrix at 45 ng/mL concentration **Figure 4**.

Figure 3. Representative chromatogram of blank HSA.

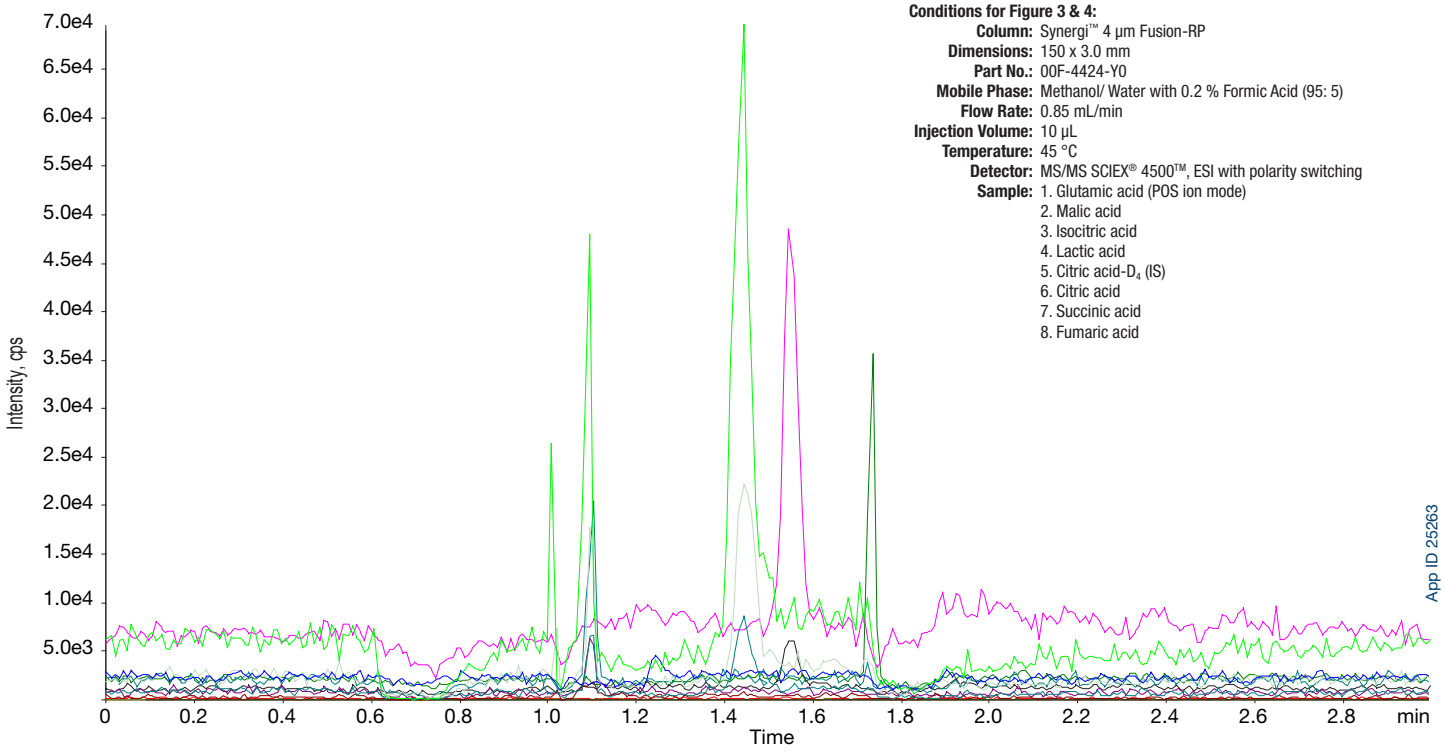


Figure 4. TCA's LLOQ (45 ng/mL) in HSA.

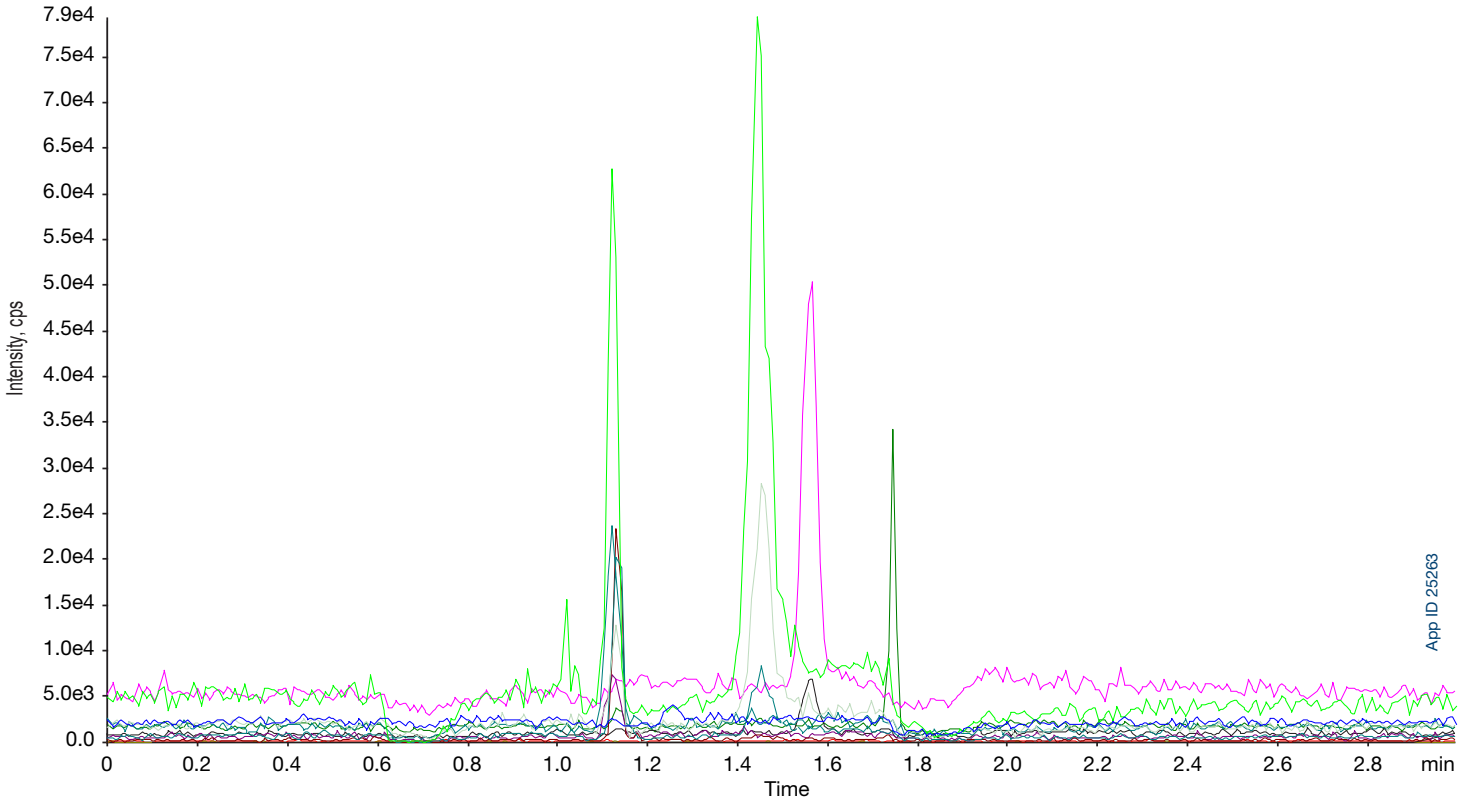


Table 3.
Summary of accuracy and precision.

Sample ID Norminal Concentration (ng/mL)	QC1 60 (ng/mL)	QC2 200 (ng/mL)	QC3 800 (ng/mL)	QC4 1500 (ng/mL)
Positive ion	Glutamic acid			
1	60.3	191	750	1550
2	53.5	184	765	1430
3	58.2	181	770	1500
4	*139	186	779	1560
5	51.4	187	783	1500
6	57.8	187	787	1520
Mean	56.2	186	772	1510
S.D.	3.66	3.35	13.6	46.5
% CV	6.51	1.8	1.77	3.08
% Theoretical	93.7	93	96.5	101
Negative ion	Citric acid			
1		*424	750	1370
2		211	*1060	1510
3		193	772	1540
4		213	752	1610
5		189	747	1390
6		201	943	1540
Mean		201	793	1493
S.D.		10.6	84.5	94
% CV		5.27	10.7	6.29
% Theoretical		101	99.1	100
Negative ion	Malic acid			
1		217	693	1410
2		208	812	1380
3		184	733	1480
4		207	702	1560
5		206	807	1480
6		174	972	1540
Mean		199	787	1475
S.D.		16.5	104	70.4
% CV		8.3	13.2	4.77
% Theoretical		100	98.3	98.3
Negative ion	Succinic acid			
1			718	1590
2			916	1390
3			898	1500
4			760	1490
5			910	1500
6			743	1470
Mean			824	1490
S.D.			93	64.2
% CV			11.3	4.31
% Theoretical			103	99.2
Negative ion	Lactic acid			
1			768	1420
2			1040	1400
3			898	1350
4			779	1610
5			791	1440
6			707	1680
Mean			821	1483
S.D.			119.8	130.6
% CV			14.4	8.81
% Theoretical			104	98.9
Negative ion	Isocitric acid			
1			908	1280
2			914	1410
3			910	1510
4			826	1440
5			783	1480
6			850	1590
Mean			865	1452
S.D.			54.3	104.6
% CV			6.28	7.2
% Theoretical			108	96.8

+ positive ion

Figure 5.
Calibration curves in the matrix for Glutamic, Malic, Succinic, Lactic, Citric, and Isocitric acid.

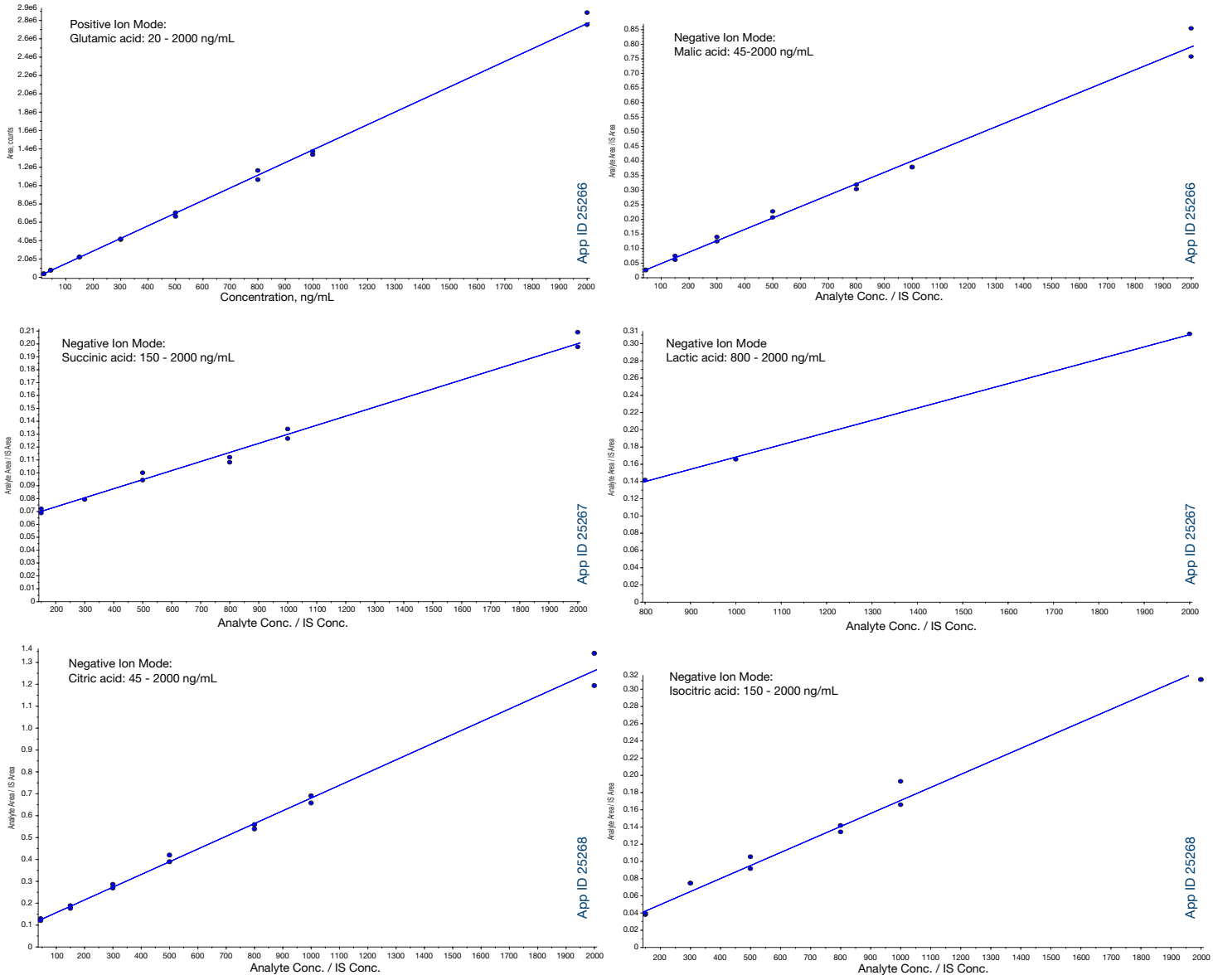


Table 4.
TCA's dynamic curve ranges LLOQ to ULOQ.

Sample ID	LLOQ (ng/mL)	ULOQ (ng/mL)
Glutamic acid	20	2000
Citric acid	45	2000
Isocitric acid	150	2000
Malic acid	45	2000
Succinic acid	150	2000
Lactic acid	800	2000

Discussion

In this technical note, we addressed numerous analytical challenges associated with the analysis of TCAs within a biological matrix. Multiple reversed phase HPLC columns were screened to determine the most applicable stationary phase to improve selectivity/retention of the TCAs and provide critical separation of the isomers citric and isocitric acid. **Figure 1** is a representative chromatogram of TCA standards with the Synergi™ 4 µm Fusion-RP 150 x 3.0 mm HPLC column showing chromatographic separation of these critical isomers. The Synergi Fusion-RP displays polar retention and selectivity due to the polar embedded groups incorporated in the stationary phase.

As for detection, both positive and negative ionization modes were used to allow a single injection assay with a total run time of 3-minutes for all compounds as seen in **Figure 1.**

Figure 2 represents extracted human serum albumin Quality Control High (QCH) standard at 1500 ng/mL concentration. It demonstrated that Fumaric acid is not stable in this biological matrix with the current extraction process but exhibits acceptable reversed phase chromatography in neat solution, as seen in **Figure 1.** For the purpose of this example, we did not include Fumaric acid in the final accuracy and precision evaluation depicted in **Table 3, Table 4, or Figure 5.**

Figures 3 and **4** are a comparison of blank human serum albumin vs a 45 ng/mL spiked human serum albumin sample, which represented the lower limit of quantification in this matrix under negative ion mode. It was observed that biological based matrices exhibited an inherently high endogenous level of TCAs, which contributed additional constraints to the linear range of this method. The human serum albumin selected for this method was fatty acid free.

By selecting an appropriate stationary phase, we were able to use an MS compatible mobile phase that did not require any derivatization or ion-pairing agents. The mobile phase was premixed prior to use due to the sensitivity of the method to the aqueous solvent ratio and ensure the solution's consistent pH. The addition of Formic acid also aided in analyte stability and chromatographic peak shape.

The reference assay demonstrated acceptable accuracy and precision under GLP guidance, evidenced in **Table 3**. In addition, the assay demonstrated a dynamic calibration curve range (**Table 4** and **Figure 5**) and verified reproducibility in 2-4 levels of Quality Control standards (**Table 3**).

Conclusions

In this technical note, we investigated a reference method for the simultaneous analysis of six tricarboxylic acids out of a human serum albumin biological matrix, utilizing LC-MS/MS under reversed phase conditions. The method used Electron Spray Ionization (ESI) source with polarity switching, an appropriate stationary phase selectivity, and a mobile phase system that did not require any derivatization or the addition of an ion-pairing agent. This resulted in a fast and reproducible assay with a 3-minute analysis time. In addition, three different batches of Synergi Fusion-RP were tested to confirm method reproducibility. Assay ruggedness was demonstrated by more than 500 injections with no chromatographic change. The assay was able to analyze all six tricarboxylic acids over a dynamic range of concentrations and demonstrated acceptable accuracy and precision under GLP guidance. However, Fumaric acid was not included in the final accuracy and precision evaluation due to stability issues in the matrix. As for the Citric, Isocitric, Malic, Succinic, Lactic, and Glutamic acid, this reference method was shown to be a fast and reproducible assay that can be easily adopted and implemented in most laboratories.

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Ordering Information

Synergi™ Fusion-RP HPLC Columns

2.5 µm High Speed Technology (HST) Columns (mm)						
Phase	**30 x 2.0**	**50 x 2.0**	**100 x 2.0**	**50 x 3.0**	**100 x 3.0**	**50 x 4.6**
Fusion-RP	00A-4423-B0	00B-4423-B0	00D-4423-B0	00B-4423-Y0	00D-4423-Y0	00B-4423-E0

4 µm Microbore and Minibore Columns (mm)								SecurityGuard™ Cartridges (mm)
Phase	**50 x 1.0**	**150 x 1.0**	**30 x 2.0**	**50 x 2.0**	**75 x 2.0**	**150 x 2.0**	**250 x 2.0**	**4 x 2.0***
Fusion-RP	00B-4424-A0	00F-4424-A0	00A-4424-B0	00B-4424-B0	00C-4424-B0	00F-4424-B0	00G-4424-B0	AJ0-7556 for ID: 2.0-3.0 mm

4 µm MidBore™ Columns (mm)				SecurityGuard Cartridges (mm)
Phase	**50 x 3.0**	**150 x 3.0**	**250 x 3.0**	**4 x 2.0***
Fusion-RP	00B-4424-Y0	00F-4424-Y0	00G-4424-Y0	AJ0-7556 for ID: 2.0-3.0 mm

4 µm Analytical Columns (mm)					SecurityGuard Cartridges (mm)
Phase	**50 x 4.6**	**75 x 4.6**	**150 x 4.6**	**250 x 4.6**	**4 x 3.0***
Fusion-RP	00B-4424-E0	00C-4424-E0	00F-4424-E0	00G-4424-E0	AJ0-7557 for ID: 3.2-8.0 mm

* SecurityGuard Analytical cartridges require holder, Part No.: KJ0-4282

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