Improving sensitivity for the quantification of TCA cycle analytes in human plasma by the ACQUITY™ Premier system solution

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INTRODUCTION

The Tricarboxylic acid cycle (TCA cycle) is a series of enzyme-mediated chemical reactions of substrates arising from the breakdown of carbohydrates, fats, and proteins (1). The TCA cycle a tightly regulated pathway that is used for both anabolic and catabolic cellular processes (1). Further, metabolites arising from its' reactions have been shown to facilitate cell signaling through the mitochondria (2). The ultimate fate of metabolites from the TCA cycle put it in a central role of cell homeostasis, and the monitoring of its constituents and products can give insights to various diseases.

The measurement of compounds such as those of the TCA cycle can be troublesome. Aside from their low molecular weight coupled with the complexity of matrix extracts as well as stability issues (3, 4), the interaction of compounds with carboxylic and phosphoric acid moieties with metal is a known complication causing loss of analytical sensitivity and poor peak shape (5, 6). The uncertainty in measurement accompanying such secondary interactions can be detrimental to the analysis. Methods of attenuation include the addition of chelators in the mobile phase (7-10) or in the sample diluent (10), however such mobile phase modifiers could contribute to ion suppression or changes in chromatography leading to the remediation of one problem, but the creation of another. To address retention and selectivity, we have developed a mixed-mode anion exchange chromatographic method using the CSH™ Phenyl-Hexyl column and simple mobile phase. To address the metal interactions, Waters has developed MaxPeak High Performance Surface (HPS) technologies (11). By utilizing this technology with LC components as well as column hardware, we are able to mitigate analyte interactions with the metal surfaces and eliminate the need for additional mobile phase additives.

INSTRUMENTAL CONDITIONS

LC System:	ACQUITY™ Premier LC
MS:	XEVO TQ-Smicro, ESI negative
Column:	ACQUITY™ Premier CSH™ Phenyl-Hexyl, 1.7 µm; 2.1 x 100 mm (p/n 186009475)
Mobile Phase A:	0.1% Formic Acid in Water
Mobiel Phase B:	0.1% Formic Acid in ACN
Column Temp:	50˚C
Injection Volume:	3 µL

Gradient Table:

Time	Flow Rate	%A	%B	Curve
initial	0.5	100	0	
0.50	0.5	100	0	6
3.50	0.5	75	25	6
3.60	0.5	0	100	6
5.00	0.5	0	100	6
5.10	0.5	100	0	6

EXPERIMENTAL

Materials

All analyte standards were purchased from Sigma Aldrich and stable isotope labeled internal standards (SIL IS) were purchased from Cambridge Isotope Laboratories. Analyte stock solutions were prepared individually at 50 mM free acid in H_2O and subsequently combined at 2.5 mM in H_2O for the working stock solution. SIL IS were each prepared at 1 mM free acid in 50%ACN/50%H_2O. A working internal standard mix of 38 µm for cis-aconitic acid-C13, 2-hydroxyglutaric acid-C13, fumaric acid-C13, malic acid-C13, succinic acid-C13 and 120 µm for citric acid-C13 was then prepared in H_2O.

Plasma Extraction

Four individual donor female healthy plasma samples as well as breast cancer positive samples were purchased from BioIVT (Westbury, NY). Following defrosting on ice, 25 µL of each plasma sample was added to a new 1.5 mL microcentrifuge tube followed by 5 µL of working internal standard mix. 75 µL of cold methanol was added and the samples vortex mixed for 1 minute. The samples were centrifuged at 21,130 rcf and 4 °C for 10 minutes. Following, 75 µL of supernatant was transferred to a new microcentrifuge tube and dried in a centrifugal evaporator under vacuum without heating for 1.5 hours. Samples were immediately reconstituted with 75 µL of H2O and placed at 20 °C for 10 minutes. Reconstituted samples were then centrifuged as above, and samples transferred to vials for analysis.

Calibration Curve Preparation

Calibration curves in H_2O were prepared by serial dilution from the working stock solution to 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 µM, with 5 µL of SIL IS stock solution added to each vial, matching the same volume as plasma samples, 25 µL. 50 µL of water was then added to make a final volume of 75 µL, matching the sample preparation and dilution procedure for the plasma samples.

RESULTS

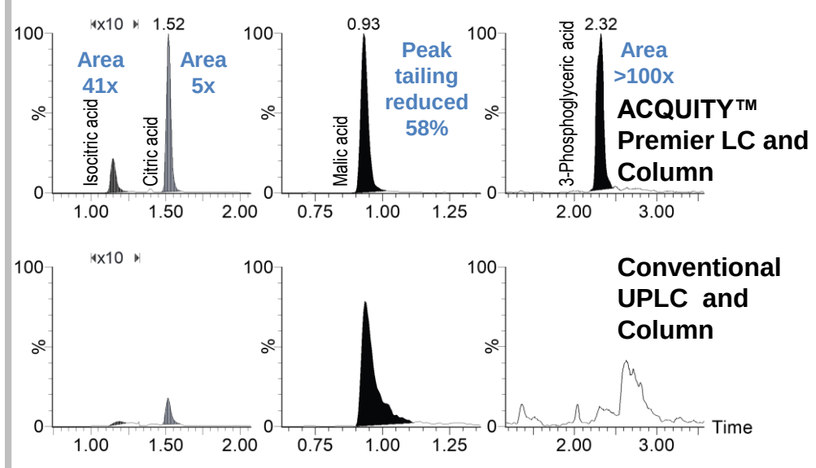

Figure 1. Increasing analyte sensitivity without additives Benefits of the MaxPeak High Performance Surfaces technology in this application include an increase in the peak area as well as improved peak shape for metal sensitive compounds. Here we see the separation of metabolites extracted from plasma on the ACQUITY™ Premier LC and Column (top) compared with a Conventional UPLC and Column (bottom). These results show an increase in peak area for isocitric and citric acids as well as 3-phosphoglyceric acid. Additionally, there is a reduction of peak tailing for malic acid.

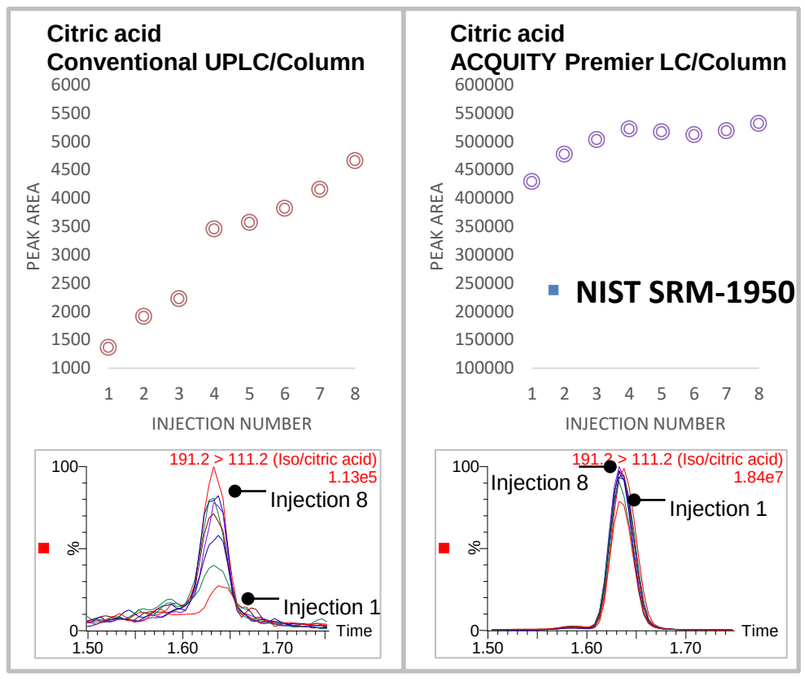

Figure 2. Improved performance out of the box Injections 1-8 for new columns out of the box using extracted plasma SRM-1950 for citric acid.

Figure 3. Calibration curves were performed for the analytes in water, using isotopically labeled internal standards where possible and linear regression with 1/x weighting.

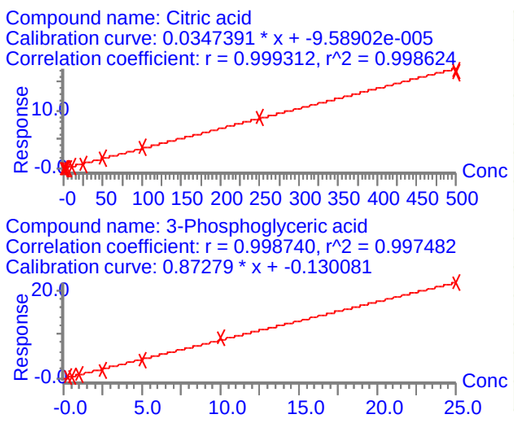

Table 1. Summary of calibration curve results. Concentrations are absolute values as measured after dilution step in sample prep.

Table 1.	Linearity Range			
Compound	Retention time (min)	Min (µm) **LLOQ**	Max (µm)	R^2 (Linear fit)
Malic acid	0.93	0.033	167	0.9991
2-Hydroxyglutaric acid	1.02	0.083	83.3	0.9969
Succinic acid	1.04	0.083	83.3	0.9995
Isocitric acid	1.15	0.083	83.3	0.9943
Citric acid	1.52	0.033	167	0.9986
Fumaric acid	1.74	0.083	167	0.9989
Pyruvic acid	2.04	1.67	83.3	0.9949
3-Phosphoglyceric acid	2.34	0.083	8.33	0.9975
α-Ketoglutaric acid	2.36	0.333	33.3	0.9981
Phosphoenolpyruvic	2.67	0.033	16.7	0.9966
cis-Aconitic acid	3.08	0.167	83.3	0.9973

Table 2.	Mean Concentration in Samples n = 3 (µm)							
Compound	Healthy Control 1	Healthy Control 2	Healthy Control 3	Healthy Control 4	Breast Cancer 1	Breast Cancer 2	Breast Cancer 3	Breast Cancer 4
Malic acid	12.2	10.9	3.93	3.67	5.43	28.3	30.7	13.6
2-Hydroxyglutaric acid	1.97	4.07	0.9	0.4	2.83	7.27	4.27	2.67
Succinic acid	14.9	27.1	3.23	3.2	6.43	54.6	29.5	41.1
Isocitric acid	3.3	2.77	1.67	1.13	2.5	3.87	4.23	4.6
Citric acid	67.7	67.5	54.9	45.4	91.7	74.5	86.6	102.3
Fumaric acid	2.03	1.57	0.400	n/d	n/d	5.77	5.37	3.10
Pyruvic acid	254	249	138	115	63.0	n/d	7.55	6.90
3-Phosphoglyceric acid	2.15	4.64	0.584	0.900	2.63	>25	19.2	13.0
α-Ketoglutaric acid	33	90.9	14.5	4.73	78.9	n/d	n/d	n/d
Phosphoenolpyruvic	BLOQ	0.517	n/d	n/d	n/d	0.386	0.15	n/d
cis-Aconitic acid	2.07	2.03	1.27	0.9	1.93	3.17	3.53	3.93

Table 2. Summary of concentrations of analytes in each of the healthy control as well as breast cancer positive plasma samples. Back-calculated concentrations of the analytes extracted from plasma were performed using the internal standard area ratio from the solvent standards.

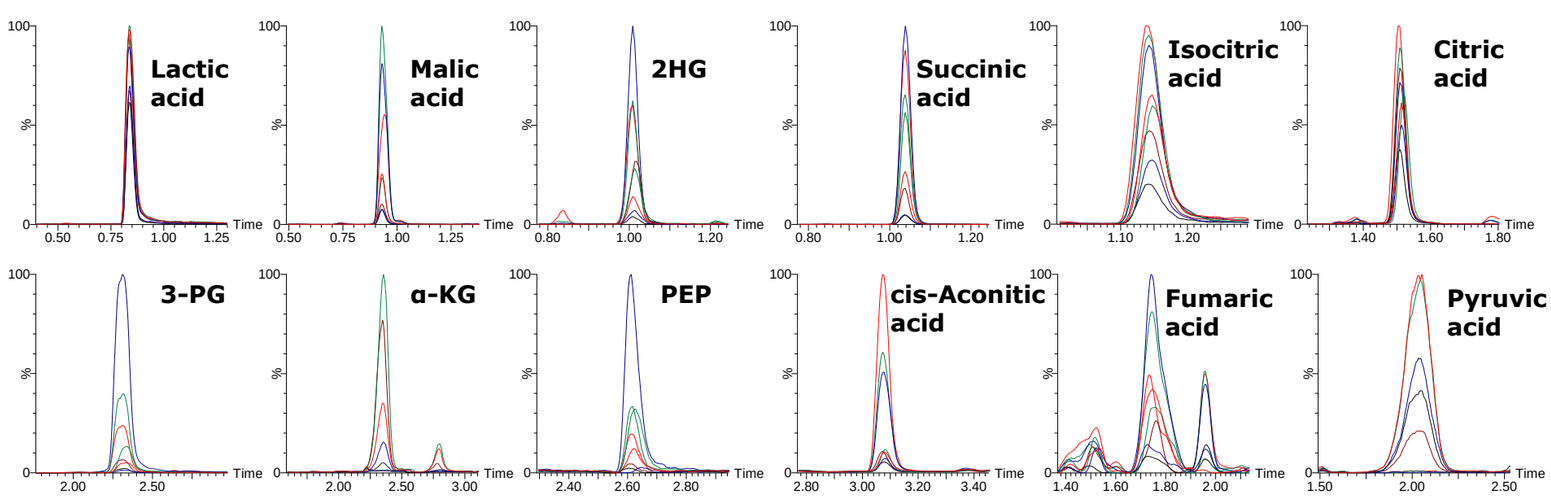

Figure 4. Overlays of analytes measured in each of the plasma samples, four control as well as four breast cancer positive, depicting separation as well as the range of concentrations measured in the plasma samples.

CONCLUSIONS

In this work, we have demonstrated that the analysis of the TCA cycle and related metabolites in human plasma can be achieved with great analytical sensitivity when incorporating MaxPeak HPS technology into the liquid chromatograph as well as the analytical column. The ACQUITY™ Premier System Solution mitigates analyte interactions with metal to improve peak shape as well as analytical sensitivity without full system passivation with strong acids or chelating additives in the mobile phase. The simple mixed-mode anion exchange separation using the ACQUITY™ Premier CSH™ Phenyl-Hexyl column allows for fast separations and easy adaptation.

- Mixed mode chromatography solved the separation challenge for the key TCA cycle analytes
- The ACQUITY™ Premier Solution gives substantial improvements in sensitivity
- Total solution reduces the hard to control, lengthy passivation process and increases the reproducibility of the assay

REFERENCES: 1. Murray RK et al. Harper's Illustrated Biochemistry, 28th ed. New York: McGraw-Hill, 2009. Chapters 16-18. Pages 131-156. **2.** Martínez-Reyes, I.; Chandel NS. Nat Commun 2020, 11 (1). **3.** Siegel, D. et al. J Chromatogr B Anal Technol Biomed Life Sci 2014, 966, 21-33. **4.** Gil, A. et al. Electrophoresis 2015, 36 (18), 2156-2169. **5.** Abrahamson, H. B. et al. Inorg Chim Acta 1994, 226 (1-2), 117-127. **6.** Sakamaki, H. et al. J Chromatogr A 2015, 1381, 125-131. **7.** Siegel, D. et al. *J Chromatogr A* 2013, *1294*, 87-97. **8.** Roberts, D. et al. *Journal of Chromatography* 1989, *471*, 437-441. **9.** Hsiao, J. J. et al. *Anal Chem* 2018, *90* (15), 9457-9464. **10.** Myint, K. et al. *Analytical Chemistry* 2009, *81*, 7766-7772. **11.** Lauber, M. et al. Waters White Paper 720006930EN. 2020.