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Applikationsbericht

Improved Analytical Sensitivity and Chromatographic Peak Shape for the Quantification of TCA Cycle Analytes in Human Plasma using the ACQUITY PREMIER System Solution

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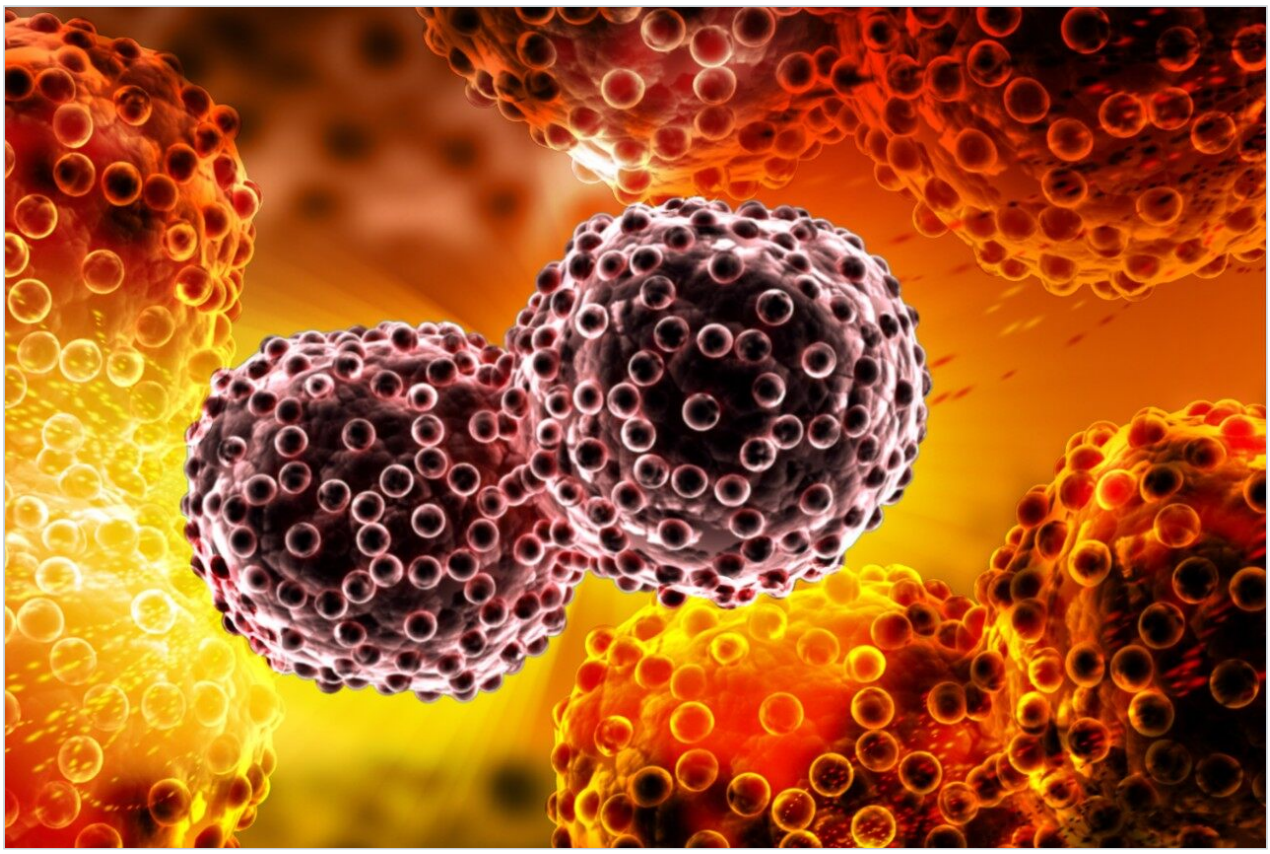

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Abstract

The analysis of TCA cycle and related metabolites in human plasma is challenging due to their small, polar nature with many isobars, wide range of concentrations in human plasma, and finally, metal sensitivity. To address retention and selectivity, the analytical method applies mixed-mode anion exchange chromatography with a simple reversed-phase like mobile phase. Tandem quadrupole mass spectrometry offers sensitive detection, while MaxPeak High Performance Surfaces mitigate interactions with metal surfaces present in the UPLC and column. The result is a simple and sensitive analytical method fit to quantify organic acid metabolites in plasma.

Benefits

- A fast and simple method for the quantification of TCA cycle and other metabolites in human plasma without complicated mobile phases
- Improved analytical sensitivity and peak shape by eliminating metal-analyte interactions using MaxPeak High Performance Surface Technology in the ACQUITY PREMIER System Solution

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, 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.[3-6] 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 or in the sample diluent, 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.[7-10] 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.

Experimental

Stock Solutions Preparation

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. Stable labelled internal standards (Cambridge Isotope Laboratories) 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 H_2O and placed at 20 °C for 10 minutes. Reconstituted samples were then centrifuged as above, and samples transferred to new silanized vials for analysis. Plasma samples were injected in triplicate.

Calibration Curve Preparation

Calibration curves in H_2O were prepared by serial dilution from the working stock solution to make stock standards of 2.5 mM, 1.25 mM, 500 µM, 250 µM, 125 µM, 50 µm, 25 µM, 12.5 µM, 5 µM, 2.5 µM, 1.25 µM, and 0.50 µM. Five microliters of each standard as well as 5 µL of the internal standard mixture was added to 15 µL of water in a silanized vial. This represents calibration solutions of 500, 250, 100, 50, 25, 10, 5, 2.5, 1, 0.5,

0.25, and 0.1 µM respectively in the same initial volume as plasma, 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. Thus, the actual concentration of the calibration curve as measured on the mass spectrometer was 167, 83.3, 33.3, 16.7, 8.33, 3.33, 1.67, 0.833, 0.333, 0.167, 0.083, and 0.033 µM. Calibration standards were injected in duplicate.

Mobile Phase Preparation

The mobile phases were prepared using a volumetric flask and formic acid accurately measured from ampules every three days to ensure method reproducibility.

LC Condition

LC system:	ACQUITY PREMIER
Vials:	Waters Total Recovery Vials, deactivated (p/n 186000385DV)
Column(s):	ACQUITY PREMIER CSH Phenyl-Hexyl 2.1 x 100 mm, 1.7 µm (p/n 186009475)
Column temp.:	50 °C
Sample temp.:	5 °C
Injection volume:	3 µL
Mobile phase A:	0.1% formic acid in H_2O
Mobile phase B:	0.1% formic acid in ACN

Gradient

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

MS Conditions

MS system:	Xevo TQ-S micro
Ionization mode:	ESI-
Capillary voltage:	0.5 kV
Desolvation temp.:	500 °C
Desolvation gas:	1000 L/hr
Cone gas:	50 L/hr
Source temp.:	150 °C

Compound	Precursor ion (*m/z*)	Fragment ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Lactic acid	89	43	20	12
Malic acid	133	115	25	11
2-Hydroxyglutaric acid	147	129	15	15
Succinic acid	117	73	20	10
Isocitric acid	191	111	20	13
Citric acid	191	111	20	13
Fumaric acid	115	71	25	7
Pyruvic acid	87	43	20	8
3-Phosphoglyceric acid	185	97	20	15
α-Ketoglutaric acid	145	101	10	8
Phosphoenolpyruvic acid	167	79	20	16
cis-Aconitic acid	173	129	20	10
Malic acid-C13	137	119	25	11
2-Hydroxyglutaric acid-C13	152	134	15	15
Fumaric acid-C13	119	74	25	7
Succinic acid-C13	121	76	20	10
Citric acid-C13	194	113	20	13
cis-Aconitic acid-C13	179	134	20	10

Table 1. Summary of MRM transitions.

Data Management

MS software: MassLynx v4.2

Results and Discussion

Measurement of the TCA and related components is achieved by a variety of LC-MS or GC-MS methods. Previously, we developed an LC-MS assay for the separation of TCA components in human urine by mixed-mode anion exchange chromatography on a standard CSH Phenyl-Hexyl Column and LC system.[12] Here we expand on that method by incorporating the ACQUITY PREMIER System and ACQUITY PREMIER CSH Phenyl-Hexyl Column. Both the ACQUITY PREMIER System and the ACQUITY PREMIER Column contain MaxPeak HPS Technologies to mitigate metal-analyte interactions.

Electron-rich moieties such as carboxylate and phosphate can act as metal chelators and cause unwanted

interactions with metal surfaces and may ultimately result in a reduction of the amount of analyte measured by the detector. Benefits of the PREMIER technology in this application include an increase in the peak area as well as improved peak shape for metal sensitive compounds. Figure 1 shows chromatograms of isocitric and citric acids, malic acid, and 3-phosphoglyceric acid from extracted plasma samples as analyzed on an ACQUITY PREMIER System with ACQUITY PREMIER Column compared with a standard UPLC and standard column. It is immediately evident of the increased peak area for isocitric and citric acids, as well as 3-phosphoglyceric acid, and the improved peak shape of malic acid. Isocitric acid and citric acid peak area increased 41x and 5x respectively while 3-phosphoglyceric acid topped >100x. Malic acid had a 58% reduction in peak tailing at 10% height. To determine the impact of PREMIER technology on system and column readiness, injections of extracted plasma were performed with new columns on each system. Using citric acid as an example, peak areas in plasma were >2 orders of magnitude on the first injection (Figure 2) demonstrating that the ACQUITY PREMIER UPLC and Column gives superior analytical sensitivity and requires less equilibration of sample matrix before analysis.

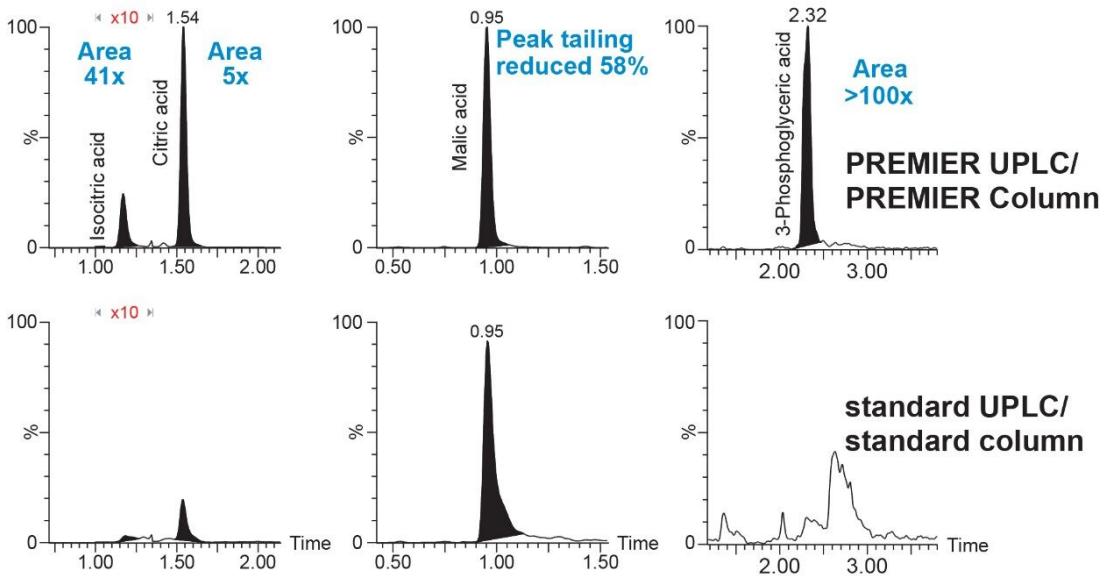


Figure 1. Separation of metabolites extracted from plasma on the ACQUITY PREMIER System and PREMIER Column (top) compared with a standard LC and standard column (bottom). These results show an increase in peak area for isocitric and citric acids as well as 3-phosphoglyceric acid. Additionally, there is a reduction of peak tailing for malic acid.

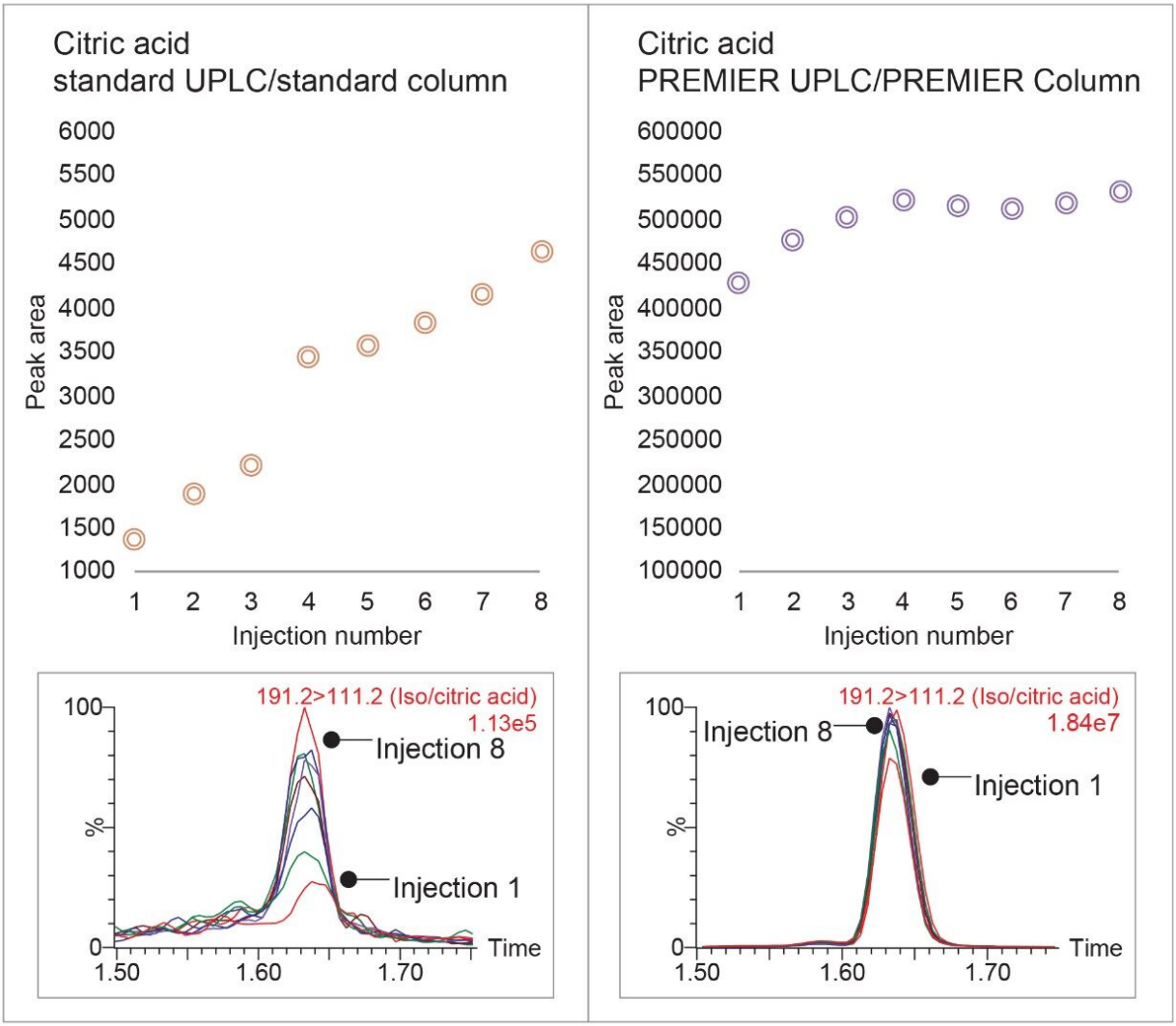


Figure 2. Injections 1–8 for new columns out of the box for extracted plasma of citric acid.

Calibration curves were performed for the analytes in water, using isotopically labeled internal standards where possible and linear regression with 1/x weighting. The calibration results are summarized in Table 2. Back-calculated concentrations of the analytes extracted from plasma were performed using the internal standard area ratio from the solvent standards (Table 3, Figure 3). A TargetLynx quantification example for citric acid is shown in Figure 4. Overlays of the analytes from each of the four healthy control as well as breast cancer positive extracted plasma samples depict the range of concentrations detected with good retention time alignment (Figure 5).

			Linearity range		
Compound	Internal standard	Retention time (min)	Min (µm) LLOQ	Max (µm)	R^2 (Linear fit)
Malic acid	Malic acid-C13	0.93	0.033	167	0.9991
2-Hydroxyglutaric acid	2-HG-C13	1.02	0.083	83.3	0.9969
Succinic acid	Succinic acid-C13	1.04	0.083	83.3	0.9995
Isocitric acid	Citric acid-C13	1.15	0.083	83.3	0.9943
Citric acid	Citric acid-C13	1.52	0.033	167	0.9986
Fumaric acid	Fumaric acid-C13	1.74	0.083	167	0.9989
Pyruvic acid	Fumaric acid-C13	2.04	1.67	83.3	0.9949
3-Phosphoglyceric acid	Fumaric acid-C13	2.34	0.083	8.33	0.9975
α-Ketoglutaric acid	Fumaric acid-C13	2.36	0.333	33.3	0.9981
Phosphoenolpyruvic acid	Aconitic acid-C13	2.67	0.033	16.7	0.9966
cis-Aconitic acid	Aconitic acid-C13	3.08	0.167	83.3	0.9973

Table 2. Summary of method and samples concentrations.

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 acid	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 3. Summary of method and samples concentrations.

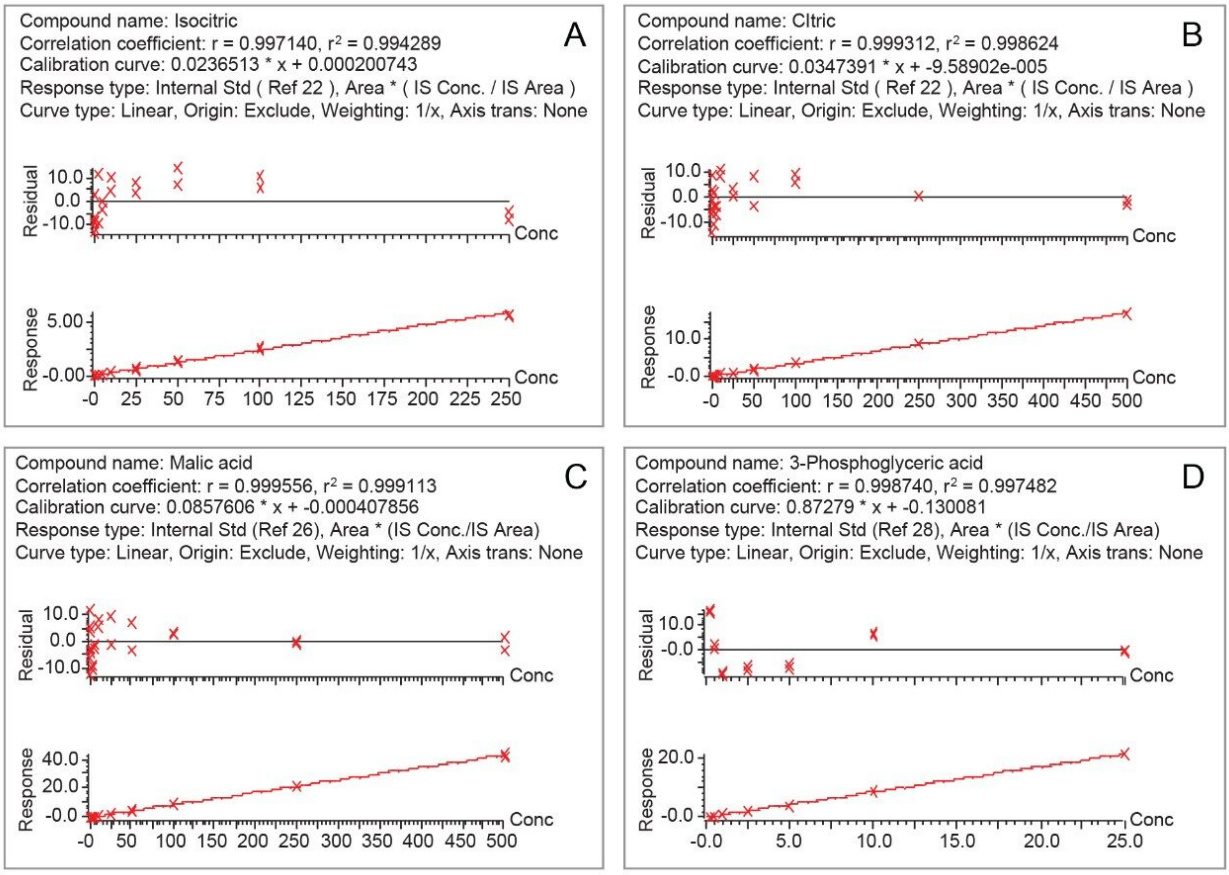
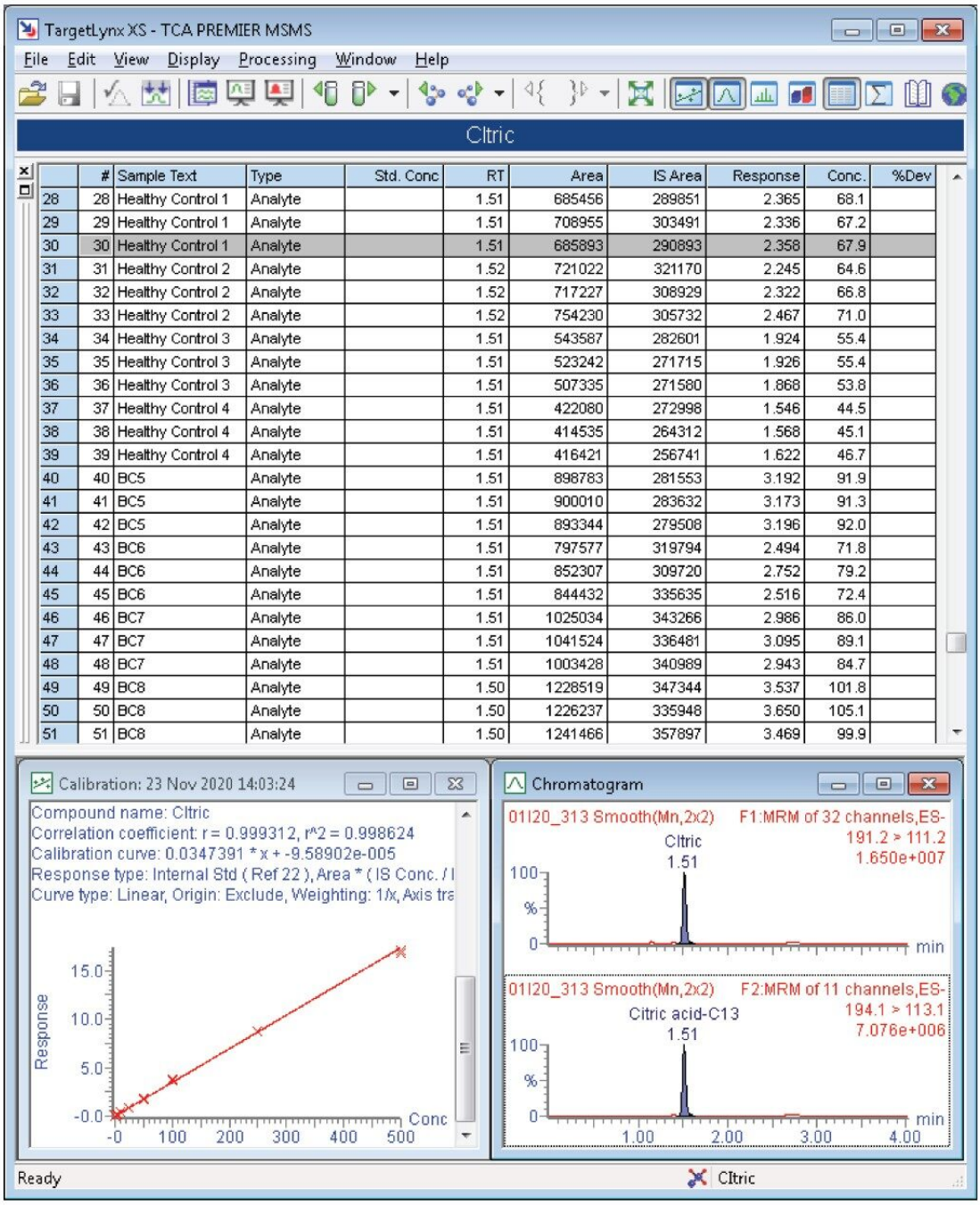


Figure 3. Calibration curves of isocitric acid (A), citric acid (B), malic acid (C), and 3-phosphoglyceric acid (D) in solution.



	#	Sample Text	Type	Std. Conc	RT	Area	IS Area	Response	Conc.	%Dev
28	28	Healthy Control 1	Analyte		1.51	685456	289851	2.365	68.1	
29	29	Healthy Control 1	Analyte		1.51	708955	303491	2.336	67.2	
30	30	Healthy Control 1	Analyte		1.51	685893	290893	2.358	67.9	
31	31	Healthy Control 2	Analyte		1.52	721022	321170	2.245	64.6	
32	32	Healthy Control 2	Analyte		1.52	717227	308929	2.322	66.8	
33	33	Healthy Control 2	Analyte		1.52	754230	305732	2.467	71.0	
34	34	Healthy Control 3	Analyte		1.51	543587	282601	1.924	55.4	
35	35	Healthy Control 3	Analyte		1.51	523242	271715	1.926	55.4	
36	36	Healthy Control 3	Analyte		1.51	507335	271580	1.868	53.8	
37	37	Healthy Control 4	Analyte		1.51	422080	272998	1.546	44.5	
38	38	Healthy Control 4	Analyte		1.51	414535	264312	1.568	45.1	
39	39	Healthy Control 4	Analyte		1.51	416421	256741	1.622	46.7	
40	40	BC5	Analyte		1.51	898783	281553	3.192	91.9	
41	41	BC5	Analyte		1.51	900010	283632	3.173	91.3	
42	42	BC5	Analyte		1.51	893344	279508	3.196	92.0	
43	43	BC6	Analyte		1.51	797577	319794	2.494	71.8	
44	44	BC6	Analyte		1.51	852307	309720	2.752	79.2	
45	45	BC6	Analyte		1.51	844432	335635	2.516	72.4	
46	46	BC7	Analyte		1.51	1025034	343266	2.986	86.0	
47	47	BC7	Analyte		1.51	1041524	336481	3.095	89.1	
48	48	BC7	Analyte		1.51	1003428	340989	2.943	84.7	
49	49	BC8	Analyte		1.50	1228519	347344	3.537	101.8	
50	50	BC8	Analyte		1.50	1226237	335948	3.650	105.1	
51	51	BC8	Analyte		1.50	1241466	357897	3.469	99.9	

Figure 4. TargetLynx results for the quantification of citric acid from healthy as well as breast cancer positive plasma samples.

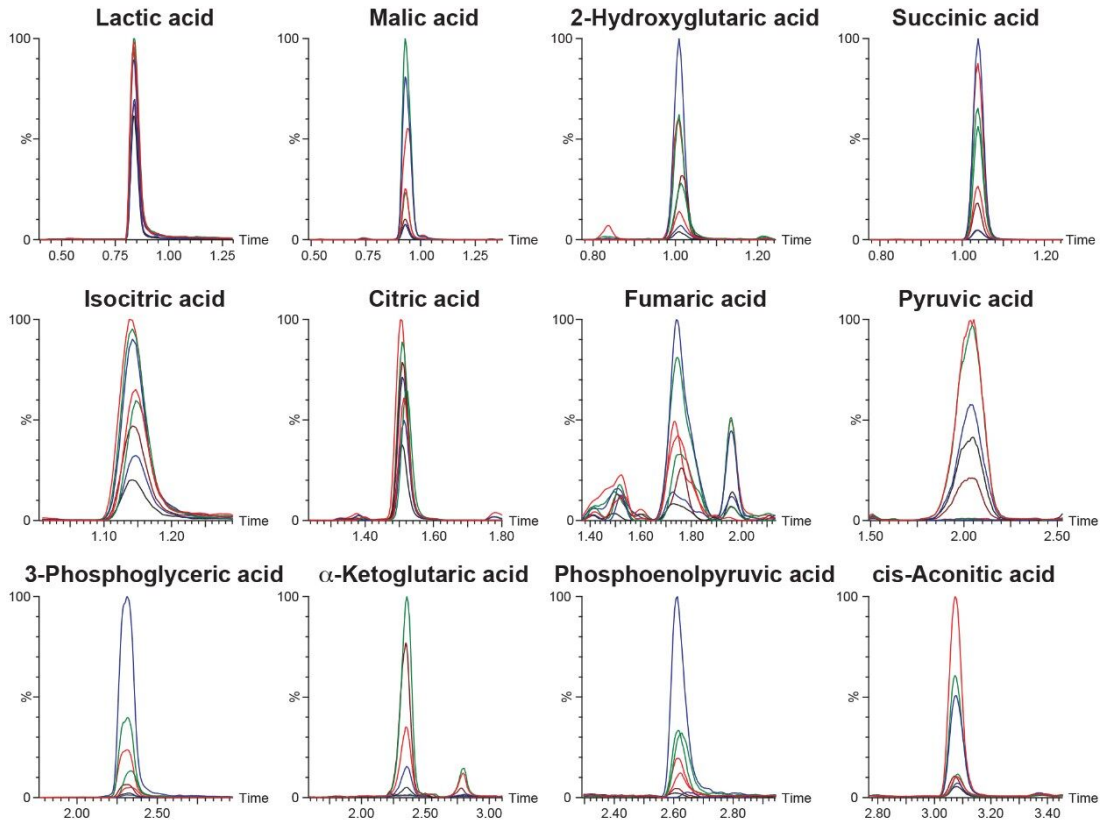


Figure 5. Overlay of analytes from the plasma samples.

Conclusion

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.

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