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Note d'application

Utilization of MaxPeak High Performance Surfaces for Improved Separation and Recovery of Analytes Associated with the Tricarboxylic Acid Cycle

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Abstract

This application note presents a mixed-mode LC method that is MS compatible for the analysis of TCA cycle analytes as well as other related compounds without the use of sample derivatization or ion-pairing reagents.

Benefits

- The use of a hybrid organic-inorganic surface technology, MaxPeak High Performance Surfaces (HPS), within the ACQUITY Premier CSH Phenyl-Hexyl Column mitigates analyte interactions with metal surfaces
- A reproducible LC-MS method for the analysis of the TCA cycle analytes as well as other related compounds without the need for sample derivatization or ion-pairing reagents
- Data processing by Progenesis QI using a custom database that includes fragment and retention time match

Introduction

The tricarboxylic acid (TCA) cycle, also known as the Krebs cycle or Citric acid cycle, is the ultimate fate of metabolism where Acetyl-CoA or other molecules are formed by the breakdown of carbohydrates, protein, and fats.[1] These molecules are then enzymatically oxidized to produce molecules such as adenosine triphosphate (ATP) to fuel cellular growth and function, as well as to reduce important cofactors that enables other metabolic processes.[1] In addition, the TCA cycle produces precursors for amino acids, proteins, fatty acids, cholesterols, and nucleotide synthesis for cell growth and division.[2]

Due to the essential metabolic processes and pathways where the TCA cycle is involved, there is a need to study the changes in the up and down regulation of analytes and how they can enable new understandings for disease states and cellular processes.[3] The components of the TCA cycle are small and polar carboxylic acids making them difficult to retain under traditional reversed-phase LC conditions. Some of the current LC methods that are utilized to analyze these compounds include: HILIC,[4] ion-pairing,[5] anion exchange,[6] and in addition derivatization followed by either gas[7] or liquid chromatography.[8] While each methodology has its own unique challenges, further complications can be caused by the presence of metal surfaces. Compounds with electron-rich moieties, such as carboxylates and phosphates, are known to chelate metals, notably iron.[9]

Here we present a mixed-mode LC method that is MS compatible for the analysis of TCA cycle analytes as well as other related compounds without the use of sample derivatization or ion-pairing reagents. The method incorporates the use of a column with hybrid organic inorganic surface technology, MaxPeak High Performance Surfaces (HPS), to mitigate analyte interactions with metal surfaces. We applied this method for the analysis of urine from healthy, and breast cancer subjects. We further used statistical software tools in order to determine any differences in analytes present as well as up- and down-regulated analytes in theses samples.

Experimental

Analysis Conditions and Sample Preparation

Four female human control and four female breast cancer positive urine samples from BioIVT (Westbury, NY) were defrosted on ice followed by 3x dilution with H_2O. The samples were then centrifuged for 10 minutes at 4 °C

and 21,130 rcf. The supernatant was transferred to a silanized total recovery vial for analysis with 50 µL of each breast cancer positive and control urine sample added to a new vial for the experimental QC. To ensure analytical performance, two system suitability samples were prepared. The first, a standard of all the analytes at a concentration of 100 µM in H_2O and a second at 100 µM spiked into a urine sample. The samples were run at the beginning and again at the end of the sample set. The samples were separated on an ACQUITY Premier CSH Phenyl-Hexyl Column using a simple mobile phase of water, acetonitrile, and 0.1% formic acid that was accurately prepared every two days. Glass ampules of formic acid were used to ensure that reproducible concentration could be obtained. The LC was connected to a Xevo G2-XS Time-of-Flight Mass Spectrometer which was operated in negative ionization mode. The Xevo G2-XS was tuned for very small molecule analysis by setting a manual profile for the quadrupole and changing the collision cell RF voltage to maximize transmission at very low *m/z*. Five replicates of each sample were randomized and then acquired in continuum format using MS^e acquisition mode of MassLynx v4.2 and further processed using Progenesis QI.

LC Conditions

System:	ACQUITY UPLC I-Class PLUS
Vials:	Waters Total Recovery, deactivated (p/n: 186000385DV)
Column:	ACQUITY Premier CSH Phenyl-Hexyl, 1.7 µm, 2.1 x 100 mm (p/n: 186009475)
Column temp.:	50 °C
Sample temp.:	10 °C
Injection volume:	3 µL
Mobile phase A:	0.1% formic acid in H_2O

Mobile phase B:	0.1% formic acid in ACN

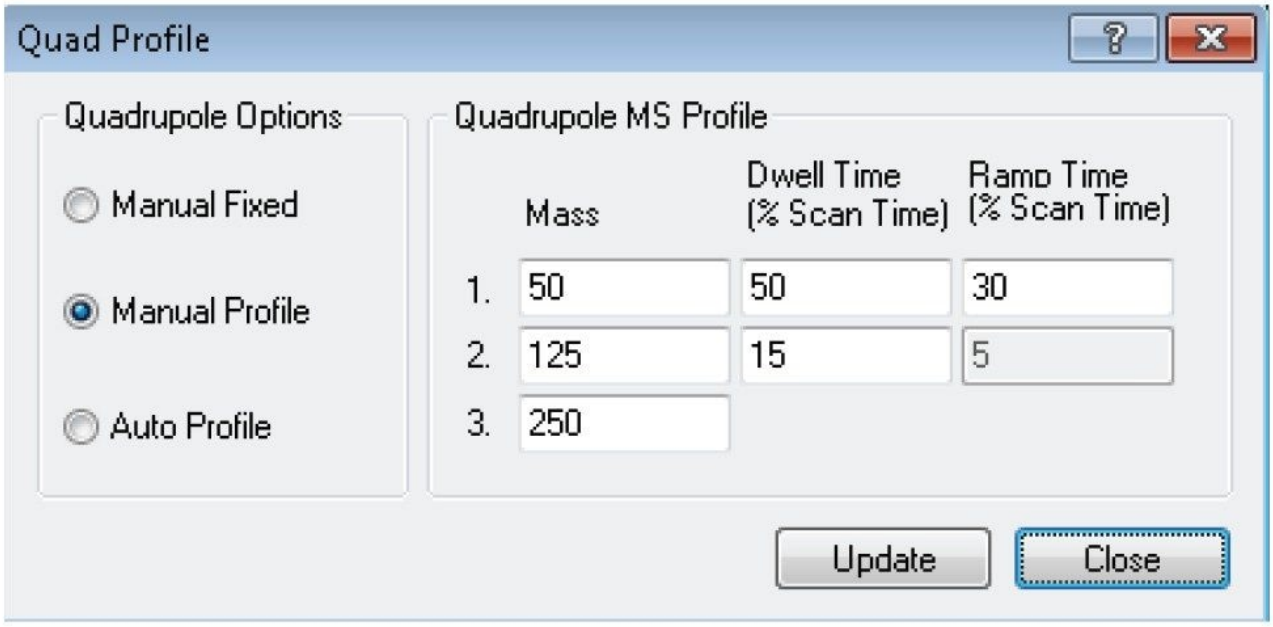
Gradient:

Time	Flow rate	%A	%B	Curve
Initial	0.4	100	0	
4.00	0.4	75	25	6
7.00	0.4	5	95	6
8.00	0.4	5	95	6
8.01	0.4	100	0	6
10.00	0.4	100	0	

MS Conditions

System:	Xevo G2-XS QTof
Ionization mode:	ESI-, resolution mode
Acquisition range:	40–950 *m/z*
Capillary voltage:	2 kV
Source offset:	50
Cone voltage:	10 V
Collision energy (low):	6
Collision energy (high):	40

Desolvation temp.:	500 °C
Desolvation gas:	1000 L/hr
Cone gas:	10 L/hr
Source temp.:	120 °C
Calibration:	Sodium formate 44.9977-928.8342 *m/z*
Quadrupole profile:	Manual profile
Lock mass:	Leucine enkephalin
Collision cell RF settings (MS mode):	Offset = 100, gain = 4
Collision cell RF settings (MS/MS mode):	Initial = 20, final = 80



Results and Discussion

The components of the TCA cycle are challenging to analyze due to their small, polar nature. Traditional methods of reversed phase chromatography do not always yield enough retention or selectivity to confidently measure these analytes. Citric acid and isocitric acid, for example, are isobaric at 191 *m/z* while malic acid undergoes degradation to have the same parent mass as fumaric acid, as discussed in reference 5. Both sets of critical pairs require chromatographic resolution for accurate determination. In a previous technology brief,[10] we showed the development of a mixed-mode separation method for the analysis of polar organic acids, including those of the TCA cycle, using tandem mass spectrometry detection. Here we expand on the method for the analysis of urinary metabolites using time-of-flight mass spectrometry. The modified method was used to analyze control and breast cancer positive urine samples. The data was subjected to multivariate analysis techniques such as principal component analysis (PCA) to statistically determine the important correlation or uniqueness of disease and non-disease samples.

Representative examples of the separation in standard as well as a pooled urine sample are shown in Figure 1, with Table 1 listing the name, retention time, as well as structure of the analytes of interest in this study. Separation was maintained for the critical pairs (Figure 2) with resolution values at full width half maximum (FWHM) of 10.9 for isocitric acid and citric acid and 32 for malic acid and fumaric acid ensuring we have confidence in their measurement.

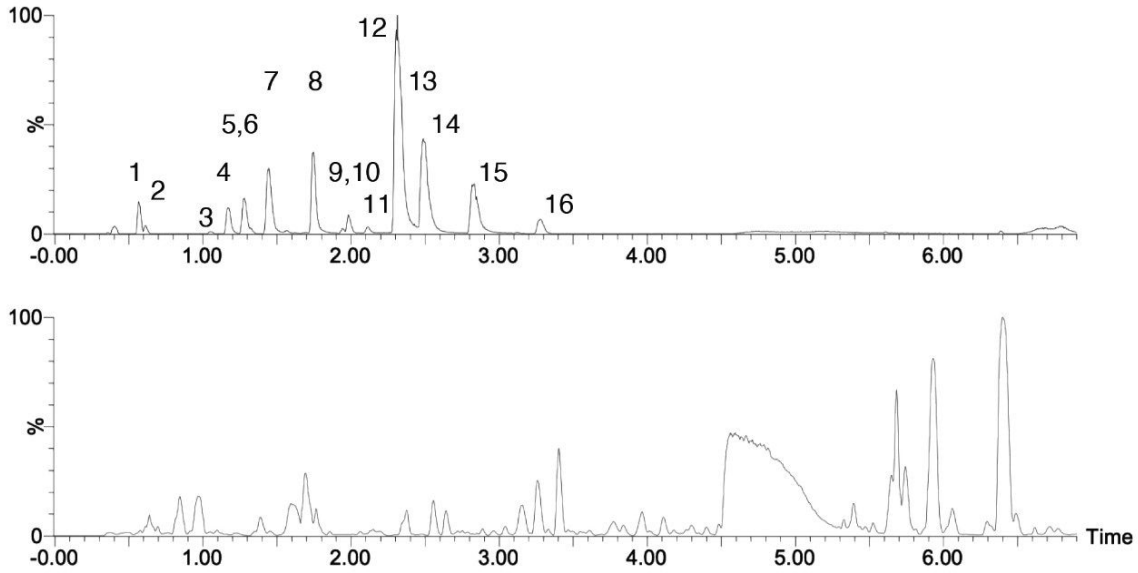


Figure 1. Separation of urinary metabolites on an ACQUITY Premier CSH Phenyl-Hexyl Column; (top) Standard mix of analytes in H_2O; (bottom) Pooled urine. See Table 1 for peak number identifiers.

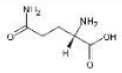
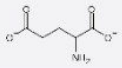
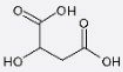
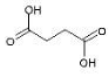
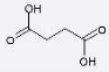
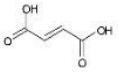
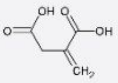
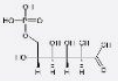
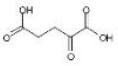
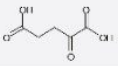
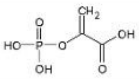
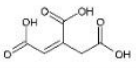
Compound name (Peak number)	Peak number	Retention time	Structure
Glutamine	1	0.62	
Glutamic acid	2	0.67	
Lactic acid	3	1.06	
Malic acid	4	1.22	
2-Hydroxyglutaric acid	5	1.31	
Succinic acid	6	1.34	
Isocitric acid	7	1.48	
Citric acid	8	1.78	
Fumaric acid	9	1.98	
Itaconic acid	10	2.02	
Pyruvic acid	11	2.33	
6-Phosphogluconic acid	12	2.47	
α-Ketoglutaric acid	13	2.64	
3-Phosphoglyceric acid	14	2.67	
Phosphoenolpyruvic acid	15	3.06	
cis-Aconitic acid	16	3.46	

Table 1. Compound names, retention times, and structures investigated in this study.

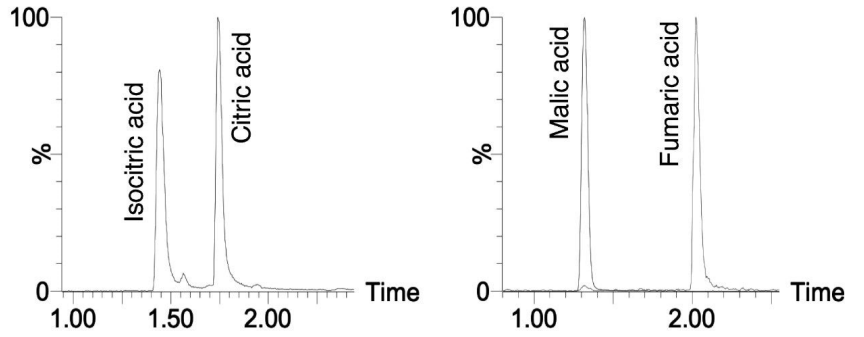


Figure 2. Separation of the critical pairs, citric/isocitric, and malic/fumaric acids.

As previously mentioned, analyte loss to metal surfaces is problematic for compounds containing electron rich moieties such as carboxylic and phosphoric acids and is especially troublesome for compounds present at low concentrations. The addition of MaxPeak HPS within the MaxPeak Premier Column family improves peak area recovery of these compounds. Figure 3 shows peak area recoveries for isocitric and citric acids, malic acid, and 3-phosphoglyceric acid on the standard CSH Phenyl Hexyl Column as well as a MaxPeak Premier CSH Phenyl Hexyl Column. In each case, the peak areas for compounds on the MaxPeak Premier Column are greater and is especially beneficial for the glycolysis intermediate, 3-phosphoglyceric acid.

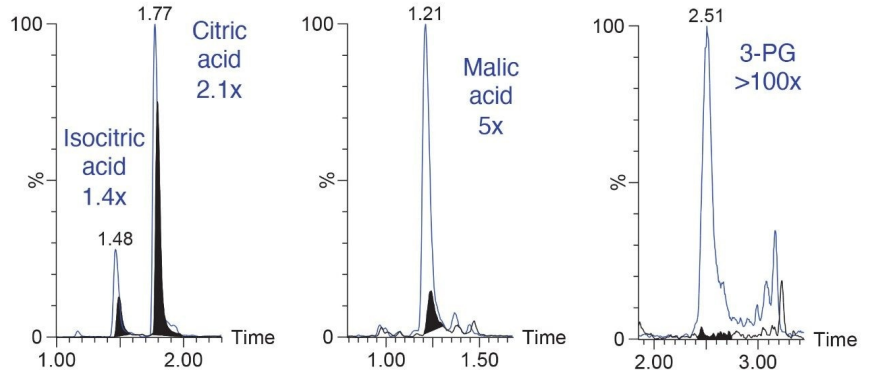


Figure 3. Peak recoveries from a urine sample for isocitric acid and citric acid, malic acid, and 3-phosphoglyceric (3-PG) acid for ACQUITY Premier CSH Phenyl Hexyl Column and a standard CSH Phenyl Hexyl Column (filled trace). Numbers show the improvement of peak area on the MaxPeak Premier Column.

The data from the analysis was then imported into and processed by Progenesis QI. The PCA scores plot in Figure 4 shows sample clusters of the five replicate injections having small variation suggesting good analytical reproducibility of the chromatographic method. Additionally, there is a clear distinction of disease vs. healthy samples. An in-house fragmentation and retention time library was created in Progenesis QI using a sample standard. Details on how to create and use a fragment database can be found in reference 11. The components of interest were then identified using this library with the constraints of mass accuracy error less than 8 ppm and retention time error less than 0.15 minutes. Finally, the component abundances for isocitric acid, citric acid, 2-hydroxyglutaric acid, 3-phosphoglyceric acid, succinic acid, and cis-aconitic acid were plotted for the breast cancer positive, control, and QC urine samples (Figure 5).

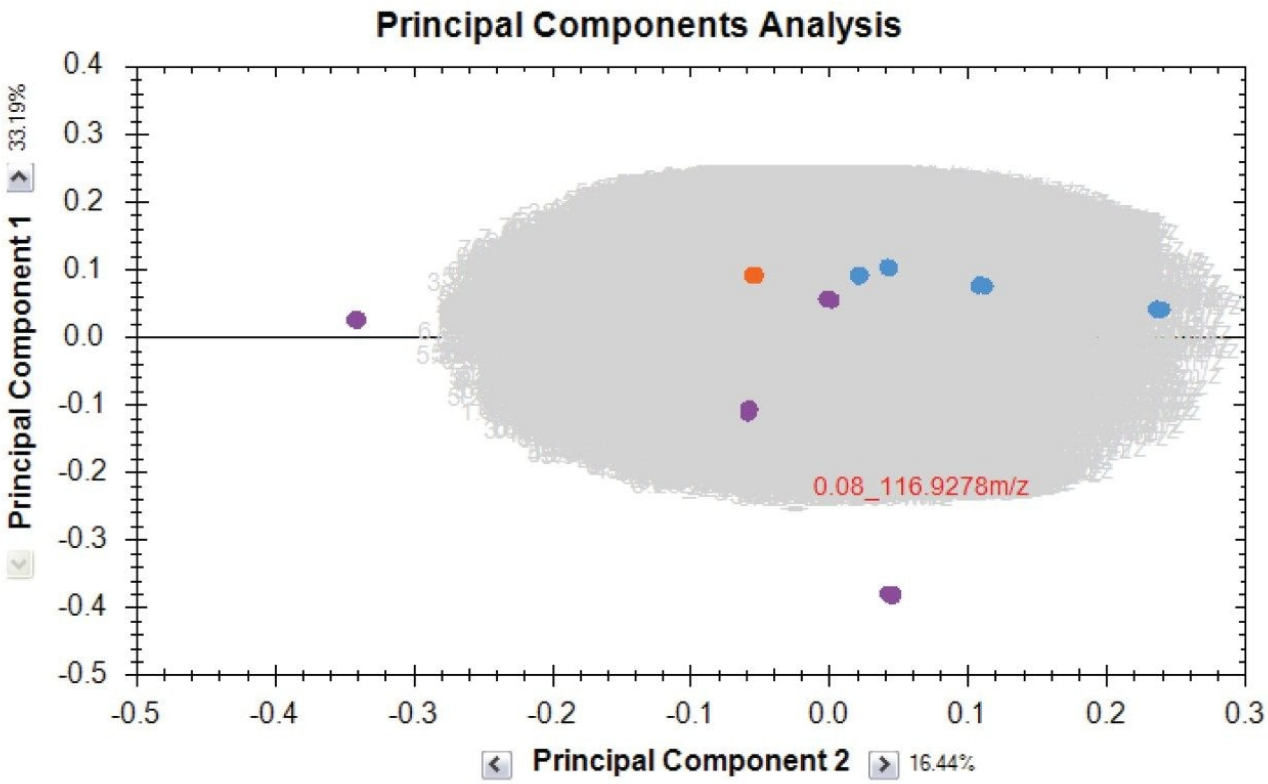


Figure 4. PCA scores plot of the study. blue: control; purple: breast cancer positive; orange: QC (pool).

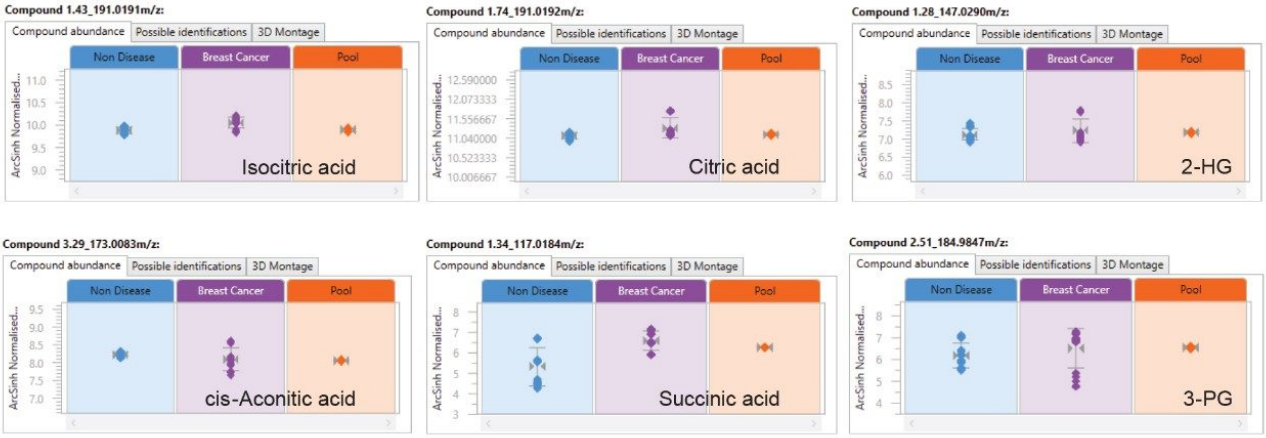


Figure 5. Abundance plots of isocitric acid, citric acid, 2-hydroxyglutaric acid (2-HG), cis-aconictic acid, succinic acid, and 3-phosphoglyceric acid (3-PG) in each of the breast cancer positive as well as the control and pooled urine (QC) injections.

Conclusion

In this work, we showed the successful application of a mixed-mode chromatographic method for the untargeted analysis of TCA analytes and other related molecules without the need for ion-pairing reagents in the mobile phase. Further, the incorporation of MaxPeak HPS within the MaxPeak Premier analytical column improves the recovery of analytes which are sensitive to metal interactions. Excellent analytical reproducibility is achieved as shown by the tight cluster of the QC samples in the PCA scores plot. The method was then used to show abundances of organic acid metabolites in both breast cancer positive and healthy urine.

References

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