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Application Note

Separation and Analysis of TCA Cycle Analytes by Mixed-Mode Chromatography Coupled with Mass Spectrometry

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Abstract

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. 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 differences in analytes present as well as up- and downregulated analytes in theses samples.

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.

Benefits

- 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 Software 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 co-factors 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 molecules that are small, polar, organic carboxylic acids that are hard to retain under traditional reversed-phase LC conditions. Some of the current LC methods utilized to analyze these compounds include HILIC,[4] ion-pairing,[5] anion exchange,[6] and derivatization, followed by either gas [7] or liquid chromatography.[8]

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. 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 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 system suitability samples were run at the beginning and again at the end of the sample set. The samples were separated on an ACQUITY UPLC 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 (Tof) 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 and further processed using Progenesis QI.

LC conditions

LC system:	ACQUITY UPLC I-Class PLUS
Vials:	Waters Total Recovery vials, Deactivated (p/n: 186000385DV)
Column:	ACQUITY UPLC CSH Phenyl-Hexyl 2.1 mm × 100 mm, 1.7 μm (p/n: 186005407)
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
Initial	0.4	100	0
4	0.4	75	25
7	0.4	5	95
8	0.4	5	95
8.01	0.4	100	0
10	0.4	100	0

MS conditions

MS 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 (high): 10–40
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*
Lock mass:	leucine enkephalin
Collision cell RF settings (MS mode):	offset=50, gain=4
Collision cell RF settings (MS/MS mode):	initial=20, final=80
Quadrupole profile:	manual profile

Data management

MS software: MassLynx, v4.2

Statistical software: Progenesis QI

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,[9] 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 healthy 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 breast cancer positive urine 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 6 for isocitric acid and citric acid, and 3.8 for malic acid and fumaric acid, ensuring we have confidence in their measurement. The data from the analysis was then imported into and processed by Progenesis QI Software.
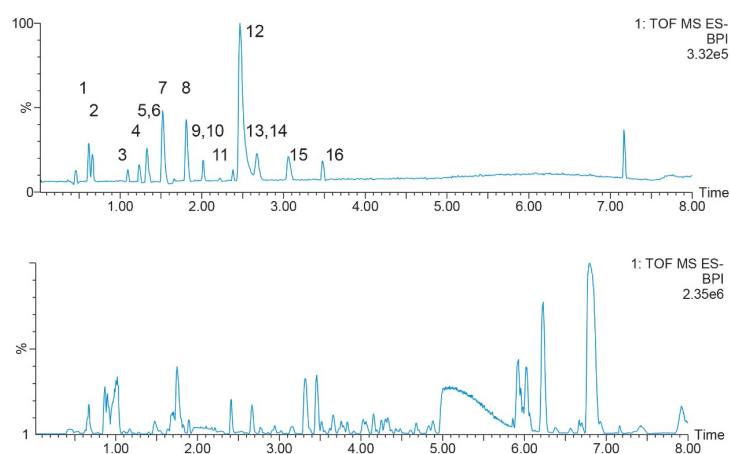

Figure 1. Separation of urinary metabolites on an ACQUITY UPLC CSH Phenyl-Hexyl Column; top, standard mix of analytes in H$_2$O; bottom, breast cancer disease sample. See table 1 for peak number identifiers.

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.
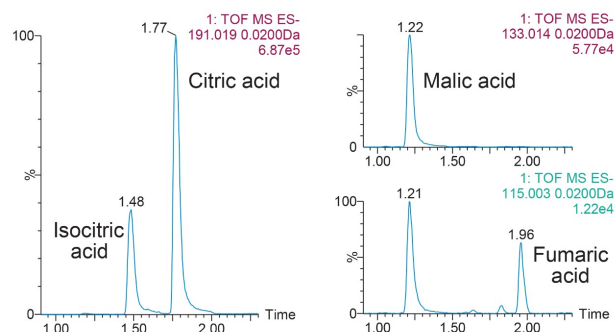

Figure 2. Separation of the critical pairs, citric/isocitric and malic/fumaric acids.

The PCA scores plot in Figure 3 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 Software using a sample standard. Details on how to create and use a fragment database can be found in reference 10. 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.
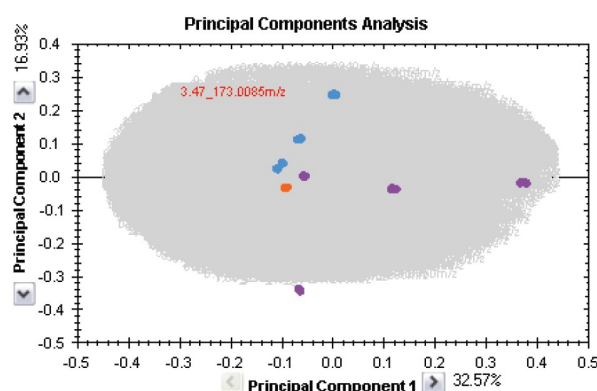

Figure 3. PCA scores plot of the study. Blue – healthy, purple – breast cancer positive, orange – QC (Pool)

An example of positively identified citric acid is shown in Figure 4. Finally, the component abundances for isocitric acid, citric acid, 2-hydroxyglutaric acid, lactic acid, succinic acid, and cis-aconitic acid were plotted for the breast cancer positive, control, and QC urine samples (Figure 5).
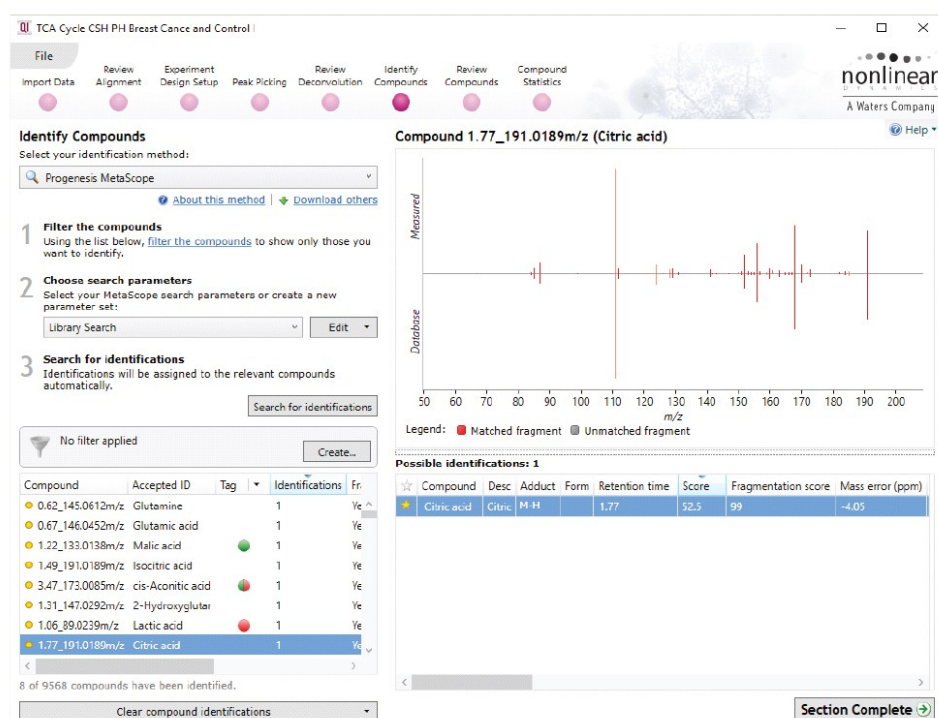
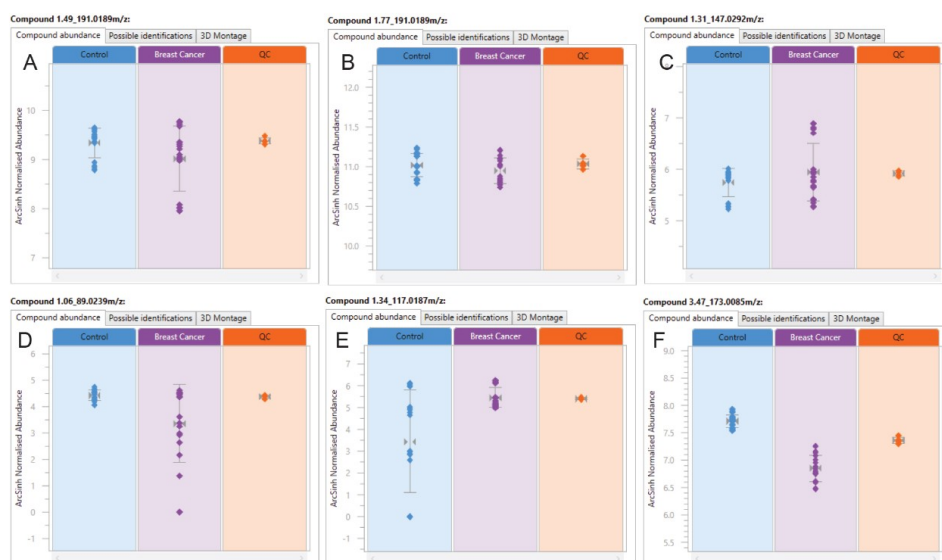Figure 4. Example of positively-identified citric acid.

Figure 5. Abundance plots of isocitric acid (A), citric acid (B), 2-hydroxyglutaric acid (C), lactic acid (D), succinic acid (E), and cis-aconitic acid (F) 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. 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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